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(71) Applicants and
(72) Inventors: **GASCHE, Christoph** [AT/AT]; Babenbergergasse 20, A-3400 Klosterneuburg (AT). **ZAKERI, Schaker, M.** [AT/AT]; Einsiedlerplatz 10/19, A-1050 Vienna (AT).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **REINISCH, Walter** [AT/AT]; Gumpoldskirchnerstrasse 26/3/17, A-2340 Mödling (AT).

(74) Agents: **SONN, Helmut** et al.; Riemergasse 14, A-1010 Vienna (AT).

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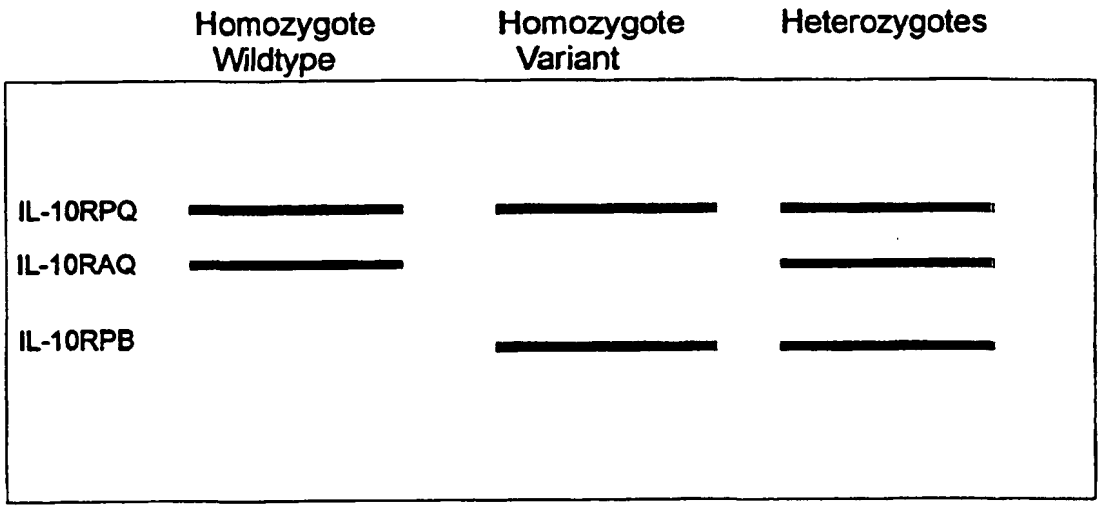
(54) Title: MAMMALIAN INTERLEUKIN-10 (IL-10) RECEPTOR VARIANTS



(57) Abstract: Amino acid sequence and nucleic acid encoding variants of the IL-10 receptors, including polymorphisms. Uses of the receptor gene and polypeptides are disclosed, including means for screening for agonists and antagonists of the receptor ligands, for producing diagnostic or therapeutic reagents, and for producing antibodies. Therapeutic or diagnostic reagents and kits are also provided.

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MAMMALIAN INTERLEUKIN-10 (IL-10) RECEPTOR VARIANTS

Field of the Invention

The present invention relates generally to nucleic acids and polypeptides characteristic of variants of receptors for mammalian interleukin-10 (IL-10). These variants preferably display at least 3 fold modified, e.g., greater, response to ligand binding than a reported receptor. The present invention also describes a single nucleotide polymorphism in the genomic sequence of the receptor that cause an incorrect splicing of the pre-mRNA and produce an aberrant mRNA which leads to a truncated and mutated form of the receptor. More particularly, this invention embraces use of variant receptors in preparing reagents useful for diagnosing or treating various IL-10 or receptor-related medical conditions.

BACKGROUND OF THE INVENTION

The proliferation, differentiation, and effector function of immune cells are regulated by a complex network of interactions. Although many of these processes involve cell-cell contact, most are mediated wholly or in part by the cytokines, a family of proteins secreted by activated hemopoietic cells. See Ho, et al. (1994) Ther. Immunol. 1:173-185. Most cytokines have more than one biological activity. The activity which is regarded as the most important likely depends on the local context in which the cytokine is produced.

As soluble intercellular messenger molecules, the cytokines typically bind to cellular receptors, e.g., cell surface receptors. Receptor molecules have been identified and isolated for G-CSF, GM-CSF, EPO, TNF, IFN-gamma, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, and many others. See, e.g., Gearing, et al. (1989) EMBO J. 8:3667-3676 (low affinity alpha chain of the human GM-CSF receptor); Itoh, et al. (1990) Science 247:324-327 (low affinity alpha chain of a mouse IL-3 receptor); Hayashida, et al. (1990) Proc. Natl Acad. Sci. 87:9655-9659 (a beta chain of a human GM-CSF receptor); Tavernier, et al. (1991) Cell 66:1175-84 (IL-5 receptor, alpha and beta chains); Silvennoinen and Ihle (1996) Signaling by the Hematopoietic Cytokine Receptors (ISBN: 041210881X); and Nicola (Ed. 1995) Guidebook to Cytokines and Their Receptors Oxford Univ. Press (ISBN: 0198599471). Many of these receptors have two chains, both of which are members of the hemopoietic receptor su-

perfamily. In such cases, typically one chain, designated the alpha chain, can bind its ligand with low affinity which may or may not result in transduction to the cell of a signal. Following the binding of a ligand to the alpha chain, another chain, designated the beta chain, is recruited and associates with the alpha chain. This interaction confers higher affinity binding of the heterodimeric receptor to the cytokine. See Miyajima, et al. (1992) Ann. Rev. Immunol. 295-331. The beta chain by itself usually lacks significant ligand binding affinity. The dimeric form of receptor is capable of transducing a signal into the cell as a consequence of ligand, e.g., cytokine, binding. Additional subunits or accessory proteins may also be associated with the receptors.

The various components of the earlier identified receptors appear to share properties useful in defining a receptor superfamily of related proteins. See Bazan (1990) Immunology Today 11:350-354; and Bazan (1990) Proc. Natl. Acad. Sci. USA 87:6934-6938. However, the structure and mechanism of action of a receptor for a mammalian interleukin-10 (IL-10) could not be predicted with reliability based merely upon speculated similarity to receptors for other cytokines.

A cytokine synthesis inhibitory factor (CSIF) activity led to assays which allowed the isolation of a cytokine designated interleukin-10 (IL-10). See Fiorentino, et al. (1989) J. Exp. Med. 170:2081-2095; and Mosmann, et al. (1991) Immunol. Today 12:A49-A53. Both mouse and human counterparts have been isolated. See Moore, et al. (1990) Science 248:1230-1234; and Vieira, et al. (1991) Proc. Nat'l Acad. Sci. USA 88:1172-1176. A human viral analog, known as either vIL-10 or BCRF1, has been described which shares many characteristic activities of the natural human form. See Hsu, et al. (1990) Science 250:830-832. Another viral homologue has been described from an equine herpes virus. See Rode, et al. (1993) Virus Genes 7:111-117.

Interleukin-10 (IL-10) inhibits cytokine synthesis by activated T cells, stimulates growth for thymocytes and mast cells, induces class II MHC expression, and sustains viability in culture of small dense resting mouse B cells (de Waal et al., 1992). Because of its inhibition of TNF production and suppression of a TH1 immune response, IL-10 has been considered as a key model for anti-inflammatory cytokines (Moore et al., 1993). Two viral IL-

10 homologues had also been identified that take advantage of the host's anti-inflammatory IL-10 signaling pathway (Liu et al., 1997a; Kotenko et al., 2000). As with other cytokines, the biological effects of IL-10 are mediated through cell-surface receptors. Human and mouse receptor subunits for IL-10 have been identified and found to be members of the interferon receptor-like subgroup of the cytokine receptor family (Tan et al., 1993; Ho et al., 1993; Liu et al., 1994). Functionally active IL-10 receptors are composed of two distinct subunits. The IL-10RA is a 110-kDa polypeptide and plays a dominant role in mediating high affinity ligand binding and signal transduction. The IL-10 receptor beta subunit (IL-10RB; also known as CRF2-4) is predicted to be a 40-kDa protein that is largely required only for signalling (Spencer et al., 1998; Kotenko et al., 1997). The human IL-10RA gene was mapped to chromosome 11q23 (Liu et al., 1994). Interaction of IL-10 with its receptors stabilizes dimerisation of both IL-10R subunits, and activates phosphorylation of the receptor-associated Janus tyrosine kinases, JAK1 and Tyk2(Stahl et al., 1995). These kinases then phosphorylate specific tyrosine residues (Y446 and Y496) on the intracellular domain of the IL-10RA. Once phosphorylated, these tyrosine residues and their flanking peptide sequences serve as temporary docking sites for the latent transcription factors, STAT1, STAT3, and in some cells also STAT5 (signal transducer and activator of transcription) (Darnell, Jr., 1997; Donnelly et al., 1999). STAT3 binds to these sites via its SH2 (Src homology 2) domain, and is, in turn, tyrosine-phosphorylated by the receptor-associated JAKs(Ullrich and Schlessinger, 1990). It then homodimerizes and translocates to the nucleus where it binds with high affinity to STAT-binding elements (SBE) in the promoters of various IL-10-responsive genes. One of these genes, SOCS-3 (Suppressor of Cytokine Signaling-3) is a member of a newly identified family of genes that inhibit JAK-STAT-dependent signaling. Studies in mice with disrupted genes for JAK1 or STAT1 have revealed that the anti-inflammatory action of IL-10 is dependent on JAK1 but does not necessarily require the presence of STAT1 (Meraz et al., 1996; Rodig et al., 1998). Serine residues of the IL-10RA (i.e. those at positions 541, 544, 553 and 554) at the carboxyl-terminal region of the murine IL-10RA are also required for inhibition of TNF-alpha production in macrophages. Thus, an accessory signal

pathway to the JAK1-STAT3 is required for expression of the anti-inflammatory actions of IL-10 (Riley et al., 1999). In contrast, the IL-10 mediated proliferation of Ba/F3 cells and activation of STAT3 is not altered by displacement of the serine residues. Indeed, STAT3 and JAK1 are central in IL-10R signaling, but not sufficient in mediating the complete repertoire of anti-inflammatory IL-10 action.

The relationship between the structure and the function of the IL-10 receptor remains incompletely understood. In order to increase the functional understanding of different receptor domains, Ho et al. created cytoplasmic receptor variants(Ho et al., 1995). Two distinct functional regions near the C-terminus of the murine IL-10RA were identified to mediate proliferation; one of these regions also mediates the differentiation response. Deletion of a third region proximal to the transmembrane domain (Aa 282-389) renders the cells 30- to 100-fold more sensitive to IL-10. The deletion mutant receptor (del 282-389) was regarded as a superactivating receptor. This deletion variant may be useful in altering the response to intervention by IL-10.

Targeted disruption of the IL-10 gene in mice causes chronic intestinal inflammation similar to human inflammatory bowel diseases (IBD, referring to both Crohn's disease and ulcerative colitis)(Kuhn et al., 1993). Silencing of other genes of the IL-10 signaling pathway showed similar intestinal inflammatory responses (Spencer et al., 1998; Takeda et al., 1999). IL-10 was shown to downregulate the enhanced protein secretion of proinflammatory cytokines in activated intestinal macrophages (Schreiber et al., 1995). A general IBD-specific alteration of the IL-10 pathway was, however, not found in human IBD subjects (Gasche et al., 2000a). The phenotypic appearance of Crohn's disease is, however, heterogenous (Gasche et al., 2000b), the genetic trait is complex, and various loci had been linked to disease susceptibility (Hugot et al., 1996; Satsangi et al., 1996; Cho et al., 1998; Hampe et al., 1999; Rioux et al., 2000). This indicates that possible genetic alterations in the IL-10 pathway in IBD may have been covered in those studies by the vast majority of IBD patients who has no genetic alteration in the IL-10 pathway.

In parallel to these investigations, the efficacy of IL-10 was tested for the treatment of IBD, specifically active Crohn's disease (Van Deventer et al., 1997; Fedorak et al., 2000; Schrei-

ber et al., 2000). The trials showed that IL-10 therapy was effective in up to 30 percent of patients, and induced persistent healing of ulcerated intestinal mucosa in some cases. These findings were remarkable, because no such endoscopic improvement was found in the placebo treated patients. Moreover, steroids, which are still regarded as the standard drug for the treatment of active Crohn's disease, do not change the degree of intestinal inflammation (Modigliani et al., 1990).

Preclinical and clinical data suggests evidence that recombinant IL-10 may be useful in the treatment of chronic hepatitis C and other chronic liver diseases that may eventually cause fibrosis and cirrhosis (Nelson, et al (2000) Gastroenterology 118: 655-660; Arai, et al (1995) J Immunol 155:5743-5749; Louis, et al (1997) Hepatology 25:1382-89).

Using SSCP analysis, Japanese investigators had identified two silent SNPs within the IL-10RA coding region (G241A and G520A do not alter the IL-10RA amino acid sequence)(Tanaka et al., 1997).

Such variants, particularly natural polymorphic forms, will also be useful to characterize regions which mediate different responses of ligand binding or to identify patient populations, e.g., polymorphic variants, which may be more or less susceptible to therapeutic intervention. The present invention provides these and the means of preparing many useful reagents.

SUMMARY OF THE INVENTION

The present invention is based in part on the surprising result that certain polymorphic variants of a "standard" wild-type mammalian IL-10 receptor can be made which are differentially sensitive to a ligand, i.e., providing a higher (or lower) signaling response upon the presence of specific amounts of ligand, than the wild-type "standard" counterpart. In particular, sites of the receptor have been identified which, upon modification, result in a differentially active receptor. As potential critical residues, these residue positions are likely able to alternatively decrease responsiveness. Here, substitution variants are identified leading to a functional phenotype.

One embodiment includes, e.g., an isolated or recombinant polypeptide comprising a sequence substitution at position gly351 and/or ser159 (according to Seq.ID No.2) or from position 62

(e.g. in Seq.ID No.5; i.e. wherein leu62 is wild type and arg63 is the first amino acid from the substituted further sequence) of standard human IL-10 receptor subunit alpha (IL-10RA). In various embodiments, the substitution of gly is with a charged amino acid; the substitution of ser is with an uncharged amino acid; the polypeptide effects at least a three fold change in signal from IL-10 binding; the substitution of gly is with arginine (especially the substitution of the AGC codon (nucleotides 475 to 477 according to Seq.ID No. 1)(preferably to GGC)); the substitution of ser is with gly (especially the substitution of the GGG codon (nucleotides 1051 to 1053 according to Seq.ID No. 1)(preferably to AGG)); or the change is an increase. Preferred embodiments of the variant polypeptides according to the present invention are the processed forms of these variant IL-10RA polypeptides, especially forms lacking the 21 amino acid long N-terminal signal sequence (see: e.g. GeneBank entry U00672).

Other embodiments include methods of making an antibody which can distinguish the IL-10RA polypeptide variant from the corresponding polypeptide of standard human IL-10RA, comprising immunizing a mammal with the variant, thereby producing an antibody which recognizes the variant but not the standard IL-10RA, e.g., comprising expressing an isolated or recombinant nucleic acid encoding the variant polypeptide. Thus, the invention also provides an isolated or recombinant nucleic acid encoding the variant polypeptide, and a cell transformed with the isolated nucleic acid.

Alternatively, the invention provides an isolated or recombinant IL-10RA polypeptide of at least 12 amino acids comprising at least three residues matching each side of and flanking a substitution of gly351 or ser159 or from position 63 of standard human IL-10RA. Preferred embodiments include, e.g., wherein the: substitution of gly351 of standard human IL-10RA is a superactivating IL-10RA; substitution is G351R or S159G; polypeptide matches at least 5 residues on each side flanking gly351 or ser159; substitution of IL-10RA effects at least a three fold change in signal from IL-10 binding; polypeptide comprising a sequence of TLGNREPPV or DTYESIFSH; or change is an increase. Similarly, the invention provides methods of making a polypeptide comprising expressing a nucleic acid encoding the polypeptide. Such an isolated or recombinant nucleic acid encoding the recom-

binant polypeptide is also provided, including, e.g., a nucleic acid further encoding other portions of the IL-10RA, e.g.,: the extracellular domain of IL-10RA; the intracellular domain of IL-10RA; N proximal sequence from S159 of IL-10RA; or C proximal sequence from G351 of IL-10RA. Also embraced is a cell transformed with the nucleic acid.

Another embodiment of the present invention relates to a substitution from position 63 onwards, i.e. leu62 being according to the human wild type sequence, the further amino acids being truncated or at least partially substituted by other amino acids, especially according to Seq.ID. No. 5. A preferred deletion relates to a polypeptide encoded by a nucleotide sequence comprising the coding sequence for standard human IL-10RA, wherein nucleotides after position 189 in Seq.ID No 1 are replaced by an alternative sequence, e.g. as defined in Seq.ID No.4.

The invention also provides methods of making an antibody which can distinguish the IL-10RA variant polypeptide from the corresponding polypeptide of standard human IL-10RA, comprising immunizing a mammal with the variant, thereby producing an antibody which can distinguish. The antibody will typically be capable of distinguishing a variant polypeptide from the corresponding polypeptide of standard human IL-10RA. In various forms, the antibody is: a polyclonal antibody preparation; an immunoselected antibody preparation; an immunodepleted antibody preparation; or a monoclonal antibody.

The invention further provides methods allowing distinguishing: a nucleic acid encoding the variant from one encoding standard human IL-10RA, the method comprising comparing the nucleic acid to one encoding the standard human IL-10RA; or a polypeptide variant from standard human IL-10RA, the method comprising comparing the polypeptides. Nucleic acid sequence used herein refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and DNA and RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. Similary, amino acid sequence as used herein refers to peptide or protein sequence. In certain embodiments, the comparing is: of nucleic acids, and the comparing is of: PCR products; or restriction fragments; or hybridization assays with oligo- or polynucleotides or derivates thereof which provide selective discrimination or of polypep-

tides, and the comparing is by: immunoassay; or evaluating cell responsiveness to IL-10. In preferred embodiments, the distinguishing: allows therapeutic prognosis; provides differential function information on said respective variants; or determines therapeutic treatment.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

OUTLINE

I. General

Genetic factors appear to contribute to virtually every human disease, conferring susceptibility or resistance, affecting the severity or progression of disease, and interacting with environmental influences. Much of current biomedical research, in both the public and private sectors, is based upon the expectation that understanding the genetic contribution to disease will revolutionize diagnosis, treatment, and prevention. Defining and understanding the role played by genetic factors in disease will also allow the non-genetic, environmental influences on disease to be more clearly identified and understood.

Analysis of DNA sequence variation is becoming an increasingly important source of information for identifying the genes involved in both disease and in normal biological processes. In trying to understand disease processes, information about genetic variation is critical for understanding how genes function or

malfunction, and for understanding how genetic and functional variations are related. Response to therapies can also be affected by genetic differences. Information about DNA sequence variation will thus have a wide range of application in the analysis of disease and in the development of diagnostic, therapeutic, and preventative strategies.

A key aspect of research in genetics is associating sequence variations with heritable phenotypes. There are several types of DNA sequence variation, including insertions and deletions, differences in the copy number of repeated sequences, and single base pair differences. The latter are the most common. They are termed single nucleotide polymorphisms (SNPs) when the variant sequence type has a frequency of at least 1% in the population. SNPs occur approximately once every 100 to 1000 bases. Because SNPs are expected to facilitate large-scale genetic association studies, there has recently been great interest in SNP discovery and detection(Halushka et al., 1999; Irizarry et al., 2000). Important examples of disease associations with SNPs are known, including the APOE*E4 allele (apolipoprotein E epsilon 4) in late-onset familial and sporadic Alzheimer disease (Saunders et al., 1993), the F5 1691G¢A allele (also known as factor V Leiden) in deep-venous thrombosis (Bertina et al., 1994), and the CKR5D32 (chemokine receptor 5) in resistance to HIV infection (Dean et al., 1996).

Different classes of polymorphisms are considered according to their sites within genes (non-coding, degenerate and non-degenerate sites). The coding-region SNPs (that have recently been termed cSNPs), which alter the amino acid sequence of the encoded protein, are most likely to influence disease and are found at lower rates and with lower allele frequencies than silent substitutions (Cargill et al., 1999). This likely reflects selection against harmful alleles during human evolution. Such nucleotide sequence variations may cause functional changes of the encoded proteins and could lead to disease development. It is also undoubtedly the case that some of the SNPs in non-coding DNA will also have functional consequences, such as those in sequences that regulate gene expression (Eskdale and Gallagher, 1995; Eskdale et al., 1998). Discovery of SNPs that affect biological function will become increasingly important over the next several years. From the large scale of SNPs in human DNA, those, which

render functional significance, need to be selected. Accordingly, a better understanding of the functional changes of SNPs, specifically of cSNPs, will help to better understand how genes function or malfunction. In addition, the response to therapies can be affected by such genetic differences. In fact, this is the very objective of our research proposal with regard to newly identified cSNPs on the Interleukin-10 receptor alpha (IL-10RA).

Standard methods for mutation detection include such as PCR amplification of specific alleles, multiplex PCR; oligonucleotide ligation assay, restriction fragments length polymorphism, single strand conformation polymorphism, heteroduplex analysis assays, allele specific hybridization, etc. and sequencing methods such as mini sequencing, sequencing by hybridization, allele specific nucleotide incorporation such as pyrosequencing (Pyrosequencing AG, Uppsala, Sweden), or such as TaqMan® genotyping assay(Applied Biosystems, Foster City, CA)and molecular beacon genotyping, strand displacement amplification assays, rolling circle amplifications methods, Invader Assay (Third Wave Technologies, Inc. Madison, WI)and fluorescent microarray-based methods (eg. from Affymetrix Inc., CA) and automated enzyme-linked immunoabsorbant assays (eg. Orchid BioScience Inc., Princeton, NJ) and mass spectometry genotyping technologies (eg. from Sequenom, San Diego, CA and Hamburg, Germany or Masscode™ system from Qiagen Genomics Inc.) and such as small DNA biochips containing electronically active electrodes coated with specific DNA probe such e-sensor™ system (Motorola´s Clinical Micro Sensor Technology division, Pasadena, CA) or using NanoChip™ system (Nanogen, San Diego, CA) etc, eg. in Cotton (1997) Mutation detection, Oxford University Press; Hawkins, et al (1997) Finding Mutations, The Basics IRL Press at Oxford University Press, Hirschhorn et al (2000) Proc. Natl Acad Sci USA; 97(22):12164-12169; Schweitzer, et la (2001) Curr Opin Biotechnol.;12(1):21-27, Shi, (2001) Clin Chem. 47(2):164-172; all of which are incorporated herein as reference. Standard optimization guidelines for PCR; multiplex PCR, refinement of primer design parameters etc., are described or referenced eg. in Innis; et al (1999) PCR Applications, Protocols for functional genomics, Academic Press.

Other novel methods for SNP detection to be used within the present invention are e.g.: Sapolsky et al. (1999) US Pat No. 5,858,659; Shuber (1997)US Pat No. 5,663,134; Dahlberg (1998) US

Pat 5,719,028; Murigneux (1998) WO98/30717; Shuber (1997) WO97/10366; Murphy et al (1998) Wo98/44157; Lander et al. (1998)WO98/20165; Goelet et al. (1995) WO95/12607 and Cronin et al (1998)WO98/30883. In addition Ligase base methods described by Barany et al (1997) WO97/31256 and Chen et al. Genome Res. 1998; 8(5):549-56; mass-spectoscropy-based methods by Monforte (1998)WO98/12355, Turano et al. (1998)WO98/14616 and Ross et al. (1997)Anal Chem. 15, 4197-202; PCR-based methods by Hauser et al (1998) Plant J. 16, 117-25; exonuclease based methods by Mundy Us Pat No. 4,656,127; dideoxy-based methods by Cohen et al. WO91/02087, Genetic Bit Analysis or GBA™ by Goelet et al WO92/15712; Oligonucleotide Ligation Assays or OLA by Landegren et al. (1988)Science 241:1077-1080 and Nickerson et al.(1990) Proc. Natl. Acad Sci. (U.S.A.)87:8923-8927; and primer guided nucleotide incorporation procedures by Prezant et al. (1992) Hum. Mutat. 1:159-164; Ugozzoli et al. (1992)GATA 9: 107-112; Nyréen et al. 1993 Anal. Biochem. 208:171-175.

The present invention relates to differentially activating IL-10 receptor (IL-10R) proteins and nucleic acids (see SEQ ID NO: 1-4) and antibodies. Differentially, e.g., super, activating receptors from other mammals, e.g., mouse, rat, pig, sheep, goat, etc., are also contemplated. This application hereby incorporates by reference USSN 08/110,683, filed on August 23, 1993; and USSN 08/424,788, filed on April 19, 1995.

Ba/F3 cells expressing recombinant IL-10R (BaF-mIL-10R) subunit alpha (also designated IL-10RA) exhibit a proliferative response to IL-10, whereas the parent Ba/F3 cells do not. See Ho, et al. (1993) Proc. Natl. Acad. Sci. USA 90:11267-11271; Liu, et al. (1994) J. Immunol. 152:1821-1829; USSN 08/110,683; USSN 08/011,066; and USSN 07/989,792. Both mIL-10 and hIL-10 stimulate BaF-mIL-10R (BaMR29a1) cells with similar specific activities of 0.5-1 x 107 unit/mg, similar to that observed for the macrophage deactivating factor/CSIF activity of IL-10. See Fiorentino, et al. (1989) J. Exp. Med. 170:2081-2095; Ho, et al. (1994) Therapeutic Immunology 1:173-185; and Moore, et al. (1993) Ann. Rev. Immunol. 11:165-190. See also USSN 08/110,683 and USSN 08/424,788, each of which is incorporated herein by reference.

Mutant mIL-10R containing various deletions of the cytoplasmic domain were prepared and stably expressed in Ba/F3 cells, along with individual tyrosine to phenylalanine (Y->F) mutations

of the four tyrosines (Y374F, Y396F, Y427F, and Y477F) in the cytoplasmic domain of the mature mouse IL-10R polypeptide. See Ho, et al. (1993) Proc. Natl. Acad. Sci. USA 90:11267-11271; and US Pat. 5,716,804. Two independent DNA clone isolates and their stable transfectants were characterized for each mutant. Ba/F3 transfectants stably expressing mutant IL-10R were tested for induction of proliferation by IL-10.

Ba/F3 transfectants expressing membrane-proximal variations, e.g., deletion mutations del282-389, del282-414, and del282-458, display a striking and unexpected property of a 1.5-2 log greater proliferative response to IL-10 compared to "standard" or wild-type human IL-10R (see USSN 08/110,683; note that the numeric referencing applied in the deletional studies refer to the mature sequence, with signal removed) and are thus termed "super-activating" variants. This property is not due to a significantly increased IL-10R expression level or binding affinity for IL-10, since these variant IL-10R exhibit ligand binding properties similar to both a non-super-activating variant (del483-559) and wild-type mIL-10R. In fact, Kd values for the mutant mIL-10R (400-600 pM) are somewhat higher than the wild type. See Ho, et al. (1993) Proc. Natl. Acad. Sci. USA 90:11267-11271; Tan, et al. (1993) J. Biol. Chem. 268:21053-21059; USSN 08/110,683; USSN 08/011,066; and USSN 07/989,792. Super-activation is probably not due to impaired IL-10R internalization, since mIL-10R del282-389 and del282-458 internalize 125I-hIL-10 at least as proficiently as wild-type mIL-10R, and actually better than the non-super-activating del433-559 mutant. These deletions define residues which may be generally important, e.g., in interactions with a suppressor factor, and those interactions may mediate functional differences also.

Two papers report natural polymorphic variations of human IL-10 receptor subunit alpha (IL-10RA). See Nakashima, et al. (1999) Rheumatology 38:1142-1144 (reporting G241A and G520A variants); and Tanaka, et al. (1997) Immunogenetics 46:439-441 (reporting silent variations). None of those have been identified as functionally different variations.

The super-activating receptors may lack a structure or domain which affects a self-inhibiting pathway in IL-10 signaling. There is evidence in IL-3R and EPO-R that there are cytoplasmic regions which, upon phosphorylation, normally downregulate re-

sponses to ligand binding. See D'Andrea, et al. (1991) Mol. Cell. Biol. 11:1980-1987; Sakamaki, et al. (1992) EMBO J. 11:3541-3549; and Sato, et al. (1993) EMBO J. 12:4181-4189.

Sensitivity describes an ability to induce proliferation, or other signaling readout, of a target cell expressing the different activating receptor. The present invention contemplates receptors with modified sensitivity, e.g., exhibiting a sensitivity of at least about 2-fold different than a wild-type receptor, generally at least about 4-fold, often at least about 7-fold, typically at least about 10-fold, usually at least about 13-fold, preferably at least about 20-fold, and in particular embodiments, at least about 30-fold or more change, e.g., increase or decrease, in sensitivity.

The described polymorphisms are analysed for differences in signal transduction, both with respect to quantity, e.g., strength of each effector signal, as well as quality of signal, or combination of effector functions. Thus, certain polymorphisms may lead to different changes in one effector function relative to another.

Super-activating receptors will be useful for testing a sample for the presence of a ligand, e.g., human IL-10 or analogs thereof, in a sample. Gene therapy indications may allow for cells more sensitive to lower doses of ligand. Kits exhibiting extremely high sensitivity are also contemplated for diagnostic purposes, including determination of patient subsets who may be more responsive to ligand, and susceptible to successful treatment. Conversely, less sensitive receptors may be similarly useful, e.g., in studying ligand-receptor interactions or for diagnosis of patients.

II. Receptor Variants

Isolated DNA encoding "standard" receptors can be readily modified, e.g., by nucleotide substitutions. Receptor variants can also be produced by either genetic engineering methods or protein synthesis techniques. See, e.g., USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; Sambrook, et al. (1989); Ausubel, et al. (1987 and supplements); Cunningham, et al. (1989) Science 243:1330-1336; O'Dowd, et al. (1988) J. Biol. Chem. 263:15985-15992; Beaucage and Caruthers (1981) Tetra. Letts. 22:1859-1862; and Lechleiter, et al. (1990) EMBO J. 9:4381-4390; each of which

is incorporated by reference. Additional methods will be apparent to a person having ordinary skill in the art in light of the teaching provided herein.

The IL-10RA G351R (numbered relative to the open reading frame, including signal sequence; corresponding to residue 330 of mature receptor) is located in the middle of a region (aa 282-389) which, when deleted in mice (del282-389), results in a superactivating receptor with a 30 to 100 fold increase in sensitivity to IL-10. Ho, et al. (1995) Mol. Cell. Biol. 15:5043-5053. The substitution of a neutral and polar amino acid (glycine) by a basic amino acid (arginine) in this sensitive part of the receptor could cause a change in receptor function. The allele contains a substitution of nucleotide 1051 G to A, resulting in the amino acid residue substitution, which correlates with therapeutic responsiveness in the inflammatory bowel disease studies described below. The IL-10RA G351R SNP might interfere with receptor dimerization, internalization, degradation, activation of the Jak/STAT signaling pathway, or with its negative regulation by cytokine-inducible SH2 (CIS) proteins. These potential receptor alterations could explain why patients expressing at least one IL-10RA G351R allele show a favorable response to rhIL-10. Another natural polymorphism appears to be at residue ser159, resulting from a substitution of nucleotide 475 A to G and after residue leu62, resulting from an aberrantly spliced form due to a G to A substitution in the acceptor site of the exon in the intron-exon boundary at position 255 of the genomic fragment Seq.ID No.3. Other natural variations have been identified, the G351R correlates with modified function.

Studies are possible to address some of the hypothesized modifications of receptor function. By altering the receptor sensitivity to autologous IL-10, these SNPs might also be involved in the genetic susceptibility to various diseases. Examples include inflammatory bowel diseases, autoimmune diseases, chronic graft-versus-host diseases, atopic disorders, sepsis, and lymphoproliferative tumors. This latter hypothesis will be addressed below.

III. Nucleic Acids

This invention contemplates use of isolated nucleic acids, e.g., DNA, which encode these different activating receptor com-

ponents for IL-10-like ligands, e.g., peptides. Also included are substantially homologous sequences or fragments thereof. As indicated above, specific embodiments have demonstrated that deletion of regions 282-389, 282-414, and 282-458 of mouse IL-10R result in the super-activating phenotype, and the G351R variation of human receptor probably has the same effect. This suggests that this phenotype may be correlated with other variations in these regions, particularly in the region of 282-389. Corresponding variations in species or allelic variants should also exhibit similar properties. General descriptions of nucleic acids, their manipulation, and their uses are provided in the following references: USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; Kanehisa (1984) Nuc. Acids Res. 12:203-213; Wetmur and Davidson (1968) J. Mol. Biol. 31:349-370; Goodnow (1992) "Transgenic Animals" in Roitt (ed.) Encyclopedia of Immunology Academic Press, San Diego, pp. 1502-1504; Travis (1992) Science 256:1392-1394; Kuhn, et al. (1991) Science 254:707-710; Capecchi (1989) Science 244:1288; Robertson (1987) (ed.) Teratocarcinomas and Embryonic Stem Cells: A Practical Approach IRL Press, Oxford; and Rosenberg (1992) J. Clin. Oncol. 10:180-99; each of which are incorporated by reference. Additional aspects will be apparent to a person having ordinary skill in the art in light of the teachings provided herein.

In particular, the present invention suggests that analysis of specific IL-10RA sequences may identify additional functional variants, including natural polymorphisms, as well as provide means to perform structure-function analysis. PCR or other means to study the specific sequences of the gene will be useful.

IV. Making Receptor

A DNA encoding a different activating IL-10 receptor is available by mutagenesis of a standard human IL-10 receptor available, e.g., in pSW8.1 (ATCC Deposit No. 69146) and USSN 08/110,683. The DNA can be expressed in a wide variety of expression systems as described in, e.g., USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; Pouwels, et al. (1985 and supplements) Cloning Vectors: A Laboratory Manual, Elsevier, N.Y.; Rodriquez, et al. (1988) (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston; Brosius, et al, (1988) "Expression Vectors Employing Lambda-, trp-, lac-, and

Ipp-derived Promoters", in Vectors: A Survey of Molecular Cloning Vectors and Their Uses, (eds. Rodriguez and Denhardt) Butterworths, Boston, Chapter 10, pp. 205-236; Okayama, et al. (19885) Mol. Cell Biol. 5:1136-1142; Thomas, et al. (1987) Cell 51:503-512; and O'Reilly, et al. (1992) Baculovirus Expression Vectors: A Laboratory Manual, Freeman & Co., N.Y.; each of which is incorporated by reference. It will be appreciated by those skilled in the art that a result of degeneracy of the genetic code, a multitude of IL-10RA-encoding nucleotide sequences, some bearing minimal homology to the nucleotide sequence of any known naturally occurring may be produced. The invention contemplates each and every possible variation of nucleotide sequence that could be made in accordance with the standard triplet genetic code as applied to the nucleotide sequence encoding the naturally occurring IL-10RA variants considered to be specifically disclosed. Codons may be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding the IL-10RA variants include the production of RNA transcripts having more desirable properties, such as greater half-life, than transcripts having from the naturally occurring sequence. Additional teachings will be apparent to a person having ordinary skill in the art in light of the teachings provided herein.

V. Receptor Isolation

The DNA described above will be useful in producing high levels of receptor materials. Many of the uses will not require purification of the materials as their expression on cells will often be sufficient. However, these expressed receptors can be purified as described in, e.g., USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; Hochuli (1989) Chemische Industrie 12:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Adsorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12:87-98, Plenum Press, N.Y.; Crowe, et al. (1992) QIAexpress: The High Level Expression & Protein Purification System QUIAGEN, Inc., Chatsworth, CA; Ausubel, et al. (eds.) (1987) Current Protocols in Molecular Biology; Deutscher (1990) "Guide to Protein Purification" in Meth. Enzymol., Vol. 182, and other

volumes in series; and manufacturers' literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond CA; each of which is incorporated by reference. Additional methods will be apparent to a person having ordinary skill in the art in light of the teachings provided herein.

VI. Receptor Analogs

Derivatives of naturally occurring different activating receptors are also encompassed by the present invention, particularly those exhibiting a feature of substantially different, e.g., increased, proliferative response to IL-10 ligand binding relative to a natural receptor. These derivatives include sequence variants, glycosylation variants, and covalent or aggregative conjugates with other chemical moieties. See, e.g., USSN. 08/110,683; USSN 08/011,066; USSN 07/989,792; Godowski, et al. (1988) Science 241:812-816; Beaucage and Caruthers (1981) Tetra. Letts. 22:1859-1862; Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory; Merrifield (1963) J. Amer. Chem. Soc. 85:2149-2156; Merrifield (1986) Science 232: 341-347; Atherton, et al. (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford; von Heijne (1992) J. Mol. Biol. 225:487-494; and Fasman, et al. (1990) Trends in Biochemical Sciences 15:89-92; each of which is incorporated by reference. Additional methods will be apparent to a person having ordinary skill in the art in light of the teachings provided herein.

VII. Antibodies

Various types of antibodies and antibody binding compositions can be raised to epitopes on different activating receptors, particularly those which may distinguish a different activating variant from "standard" natural form. For example, specific antigenic peptides with variant sequences in the designated region will present epitopes distinct from the "standard" sequence, e.g., as described in USSN 08/110,683. See also, e.g., USSN 08/011,066; USSN 07/989,792; Microbiology, Hoeber Medical Division, Harper and Row, 1969; Landsteiner (1962) Specificity of Serological Reactions, Dover Publications, New York; Williams, et al. (1967) Methods in Immunology and Immunochemistry, Vol. 1, Academic Press, N.Y.; Stites, et al. (eds.) Basic and Clinical

Immunology (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH Press; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, N.Y.; Kohler and Milstein (1975) in Nature 256: 495-497; Huse, et al. (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281; Ward, et al. (1989) Nature 341:544-546; U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; 4,366,241; and Cabilly, U.S. Patent No. 4,816,567; each of which is incorporated by reference. Additional methods will be apparent to a person having ordinary skill in the art in light of the teachings provided herein.

VIII. Other Uses of Receptors

Different activating receptors or fragments thereof will have many other uses, e.g., as carriers for a ligand, agonist, or antagonists; means to isolate other subunits of the receptor; in diagnostic assays; or as reagents for structural determination of critical residues in various interactions with hypothesized inhibitory interactions. See, e.g., USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; Hayashida, et al. (1990) Proc. Natl Acad. Sci. USA 87:9655-9659; Fodor, et al. (1991) Science 251:767-773; Parce, et al. (1989) Science 246:243-247; Owicki, et al. (1990) Proc. Nat'l Acad. Sci. USA 87:4007-4011; Lowenstein, et al. (1992) Cell 70:705-707; and Blundell and Johnson (1976) Protein Crystallography, Academic Press, N.Y. Diagnostic measurements other than proliferation are also contemplated, e.g., inhibition of cytokine production or induction of antigen expression. Assays include those which detect induction of transcription factors and/or DNA binding proteins, e.g., stat1 or p91. See, e.g., Pearse, et al. (1991) Proc. Natl. Acad. Sci. USA 88:11305-11309; and Pearse, et al. (1993) Proc. Natl. Acad. Sci. USA 90:4314-4318. Also contemplated are assays for molecules involved in signal transduction, e.g., phosphorylation of tyrosine kinases. See, e.g., Larner, et al. (1993) Science 261:1730-1733; and Lehmann, et al. (1994) J. Immunol. 153:165-172; each of which is incorporated by reference.

IX. Ligands: Agonists and Antagonists

The blocking of physiological response to IL-10-like peptides may result from the inhibition of binding of the ligand to the receptor, likely through competitive inhibition. Thus, in vitro assays of the present invention will often use isolated membranes from cells expressing a different activating receptor or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of either binding segment mutations and modifications, or ligand mutations and modifications, e.g., ligand analogs. See, e.g., USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; each of which are incorporated by reference.

X. Kits

This invention also contemplates use of the different activating variant IL-10 receptors, peptides, and their fusion products in a variety of diagnostic kits and methods, e.g., for detecting the presence of a ligand in a sample, e.g., mIL-10, hIL-10, or vIL-10. See, e.g., USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; U.S. Pat. No. 3,645,090; U.S. Pat. No. 3,940,475; Rattle, et al. (1984) Clin. Chem. 30(9):1457-1461; U.S. Pat. No. 4,659,678; and Rattle, et al. (1984) Clin. Chem. 30(9):1457-1461; each of which is incorporated by reference. One method for determining the concentration of IL-10 in a sample would typically comprise the steps of: expressing a transfected super-activating receptor on the surface of the host cell; contacting this cell with a sample containing the ligand, e.g., IL-10; and assaying for a biological effect, e.g., proliferation. The high sensitivity of the cells to a ligand can form the basis of a very sensitive assay.

In particular, since individuals who are more sensitive to lower levels of ligand may be more responsive to IL-10 treatment, diagnostic methods to phenotype potential target patients will be useful to evaluate and identify patients who may possess polymorphic variant from the "standard" IL-10 receptor sequence, and will respond to drug. Conversely, diagnosis of polymorphic variations may identify other subsets of patients who may be susceptible to other treatments, e.g., may exhibit lesser negative effects to ligand, or who will be not responsive to a proposed treatment. Thus, diagnosis of patients, including analysis of genotype, may become important in strategic decisions for treat-

ment.

A preferred aspect of the present invention relates to a kit for performing a method according to the present invention, i.e. for detecting a IL-10RA variant of for distinguishing said variant from standard IL-10RA comprising

- means for detecting, especially amplifying, nucleic acids encoding standard human IL-10 RA,
- means for detecting, especially amplifying, nucleic acids encoding a polypeptide variant according to the present invention,
- means for performing detection, especially amplification, of said nucleic acids and,
- optionally, means for detecting the amplified nucleic acids.

XI. Therapeutic Applications

This invention provides reagents with significant therapeutic or diagnostic value. Some polymorphic variant forms of IL-10 receptor, fragments thereof and antibodies thereto, along with compounds identified as having binding affinity to the IL-10 receptor, will be more or less susceptible to treatment relative to the standard IL-10RA. See, e.g., autoimmune conditions, septic and toxic shock conditions, and infectious conditions. See, e.g., USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; Hsu, et al. (1992) Int'l. Immunol. 4:563-569; de Waal Malefyt, et al. (1991) J. Exp. Med. 174:1209-1220; Fiorentino, et al. (1991) J. Immunol. 147:3815-3822; Ishida, et al. (1992) J. Exp. Med. 175:1213-1220; Harada, et al. (1992) J. Biol. Chem. 267:22752-22758; Gilman, et al. (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 9th ed., Pergamon Press; Remington's Pharmaceutical Sciences, 17th ed. (1992), Mack Publishing Co., Easton, Penn.; and The Merck Index, Merck & Co., Rahway, New Jersey; each of which is incorporated by reference.

XII. Additional Receptor Subunits

Various approaches would be useful for screening for accessory subunits of the IL-10 receptor. These approaches include both physical affinity methods, and activity screening. See, e.g., USSN 08/110,683; USSN 08/011,066; USSN 07/989,792; Kitamura, et al. (1991) Cell 66:1165-1174; and Hara, et al. (1992)

EMBO J. 11:1875-1884; each of which is incorporated by reference. The IL-10 receptor subunit beta (IL-10RB) has been identified. See Kotenko, et al. (1997) EMBO J. 16:5894-5903; and Spencer, et al. (1998) J. Exp. Med. 187:571-578; each of which is incorporated herein by reference.

The scope of this invention is best understood with reference to the following examples, which are not intended to limit the invention to specific embodiments exemplified.

EXPERIMENTAL

EXAMPLE 1: General Methods

Some of the standard methods are described or referenced, e.g., in Maniatis, et al. (1982) Molecular Cloning, A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual (2d ed.), vols. 1-3, CSH Press, NY; Ausubel, et al., Biology, Greene Publishing Associates, Brooklyn, NY; or Ausubel, et al. (1987 and Supplements) Current Protocols in Molecular Biology Greene/Wiley, New York; Dieffenbach, et al (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview N.Y.; all of which are each incorporated herein by reference. Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel, et al. (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification" in Methods in Enzymology vol. 182, and other volumes in this series; and manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond, CA; which are incorporated herein by reference. Combination with recombinant techniques allow fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See, e.g., Hochuli (1989) Chemische Industrie 12:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12:87-98, Plenum Press, N.Y.; and Crowe, et al. (1992) QIAexpress: The High Level Expression & Protein Purification System QUIAGEN, Inc., Chatsworth, CA: which are incorporated herein by reference.

Cell lines and tissue culture, fluorescence activated cell sorting (FACS), cytokines and antibodies, binding assays and Scatchard analysis, construction of deletion mutants, variants, IL-10 internalization, proliferation assays, DNA binding assays, and tyrosine phosphorylation studies are described, e.g., in Moore, et al., US Pat. 5,716,804.

IL-10RA variant expression vector constructs

The coding sequence of the IL-10RA cDNA was obtained by the 5'-RACE method from different individuals. These were reamplified with modified primers:
(IL-10R-EcoRV (SEQ ID NO: 6):
5'-CGATATCAGGATGCTGCCGTGCCTCGTAG-3' and
IL-10R-EcoRI (SEQ ID NO: 7):
5'-GGAATTCTCTCAGCCCGAGTCACTCAC-3', (harbouring nt59-nt1810 according to Seq ID No: 40)
for subsequent cloning. Amplicons were digested with the Eco-RV and Eco-RI and subcloned after gel purification in the expression vector pIRES-puro (Clontech Laboratories, Palo Alto, CA). Plasmids generated by this method were digested with several restriction enzymes to confirm the proper orientation and ligation of the inserts. The expression plasmids carrying the different IL-10RA haplotypes were designated for wildtype: pIRESpuro-10R1/3 and for the variant IL-10RA G351R: pIRESpuro-10R2/11. The presence of the different IL-10RA haplotypes was confirmed by Bidirectional PCR Amplification of Specific Alleles (BiPASA). To exclude the presence of an another PCR-induced mutation in insert DNA, both plasmids are subjected to dideoxy sequencing. Similar primers can be developed for other variant forms, using flanking sequences, from the coding or adjacent sequences.

Cells and Culture

Ba/F3, a murine pro-B cell line, were obtained from DSMZ (Braunschweig, Germany) and are maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine, 1 mM sodium pyruvate, 50 µg/ml gentamicin, and 10 ng/ml muIL-3.

TF-1, a human erythroleukemia cell line (DNAX, Palo Alto, CA) is grown, e.g., in RPMI 1640 supplemented with 10% fetal calf serum, 50 µg/ml gentamicin, and 1 µg/ml recombinant GM-CSF. Stable transfectants are generated, e.g., with 50 µg Nru-linearized

plasmid by electroporation (BaF3) at 400V and 960 µF on a Bio-Rad gene pulser or with 10 µg plasmid by cationic liposomal transfection (TF-1) with DMRIE (GIBCO-BRL, Gaithersburg). Each cell line is transfected with expression plasmids, e.g., pIRESpuro-10R1/3, pIRESpuro-10R2/11, and the empty vector pIRES-puro as negative control. Selection with 2 µg/ml puromycin is begun 48 h after transfection. Puromycin-resistant hIL-10RA or hIL-10RA G351R expressing clones of BA/F3 and TF-1 are isolated, e.g., sorted twice by FACS based on their expression of hIL-10RA using 3F9 mAB (DNAX) and expanded in culture.

HepG2, a human hepatocellular carcinoma cell line, is maintained, e.g., in DMEM/F12 (Gibco-BRL) supplemented with 10% fetal calf serum and 50 µg/ml gentamicin. Expression vectors of hIL-10RA are introduced into cells, e.g., by calcium phosphate-mediated transfection with Nru-linearized pIRESpuro-10R1/3, pIRESpuro-10R2/11, and pIRES-puro as control. Stable transfectants are selected, e.g., 48 hours after transfection with 1 µg/ml puromycin. Puromycin-resistant clones of HepG2 are sorted twice by FACS based on their expression of hIL-10RA using 3F9 mAB and expanded in cell culture. Scatchard plot analysis is performed as described above to verify the surface expression/cell of the two different recombinant variants of IL-10RA on transfectants.

EBV immortalized lymphoblastoid cells are grown in RPMI 1640 with L-glutamine [2 mM] and 10% FCS after incubation of PBMC with B95-8 cell supernatants (ATCC: CRL 1612, as source of EBV).

Ligand binding studies

Transfectants BAF-10R1/3, BAF-10R2/11, and TF1-10R1/3, TF1-10R2/11 are tested for specific binding, e.g., using 125I-labeled recombinant human IL-10 (DuPont-NEN, Boston, MA). 1 x 10^5 to 5 x 10^5 cells are centrifuged at 200 x g and washed with binding buffer (RPMI 1640 with 2% BSA and 0.02% sodium azide) and resuspended in 200 µl binding buffer as triplicate samples with 4-530 pM 125I-hIL-10 in the presence and absence of 100 nM hIL-10. After incubation at 4° C for 4 h in a rotary mixer, cells are pelleted through a 1:1 mixture of dibutyl- and dioctylphtahalate oils in elongated centrifuge tubes, centrifuged at 400 x g for 5 min at 4° C and quick frozen in liquid nitrogen. The cell pellet and supernatant are assessed for bound and free 125I cpm, respectively. Nonspecific binding cpm in samples containing unlabeled

hIL-10 is subtracted to obtain specific binding cpm. The dissociation constants (Kd) and the maximal concentration bound to cells (Bmax) are calculated by Scatchard analysis of the saturating binding data using linear regression analysis and replotted, e.g., with the program SigmaPlot (SPSS Inc., Chicago).

Proliferation assay

The responsiveness of the transfectants BAF-10R1/3, BAF-10R2/11, TF1-10R1/3, TF1-10R2/11, and control transfectants to various doses of hIL-10 (0.01-100 ng/ml) is tested, e.g., in triplicates after 48 h of incubation by a colorimetric cell proliferation assay using tetrazolium salt XTT (Molecular Roche, Mannheim). In brief, transfectants of BA/F3 are washed twice with in supplemented RPMI 1640 that lack mIL-3. The cells are seeded, e.g., in 96 well at a density of 2 x 104 cells/well and rested for 3 h in the absence of muIL-3. The cells are then incubated with varying amounts of hIL-10 in a total volume of 150 µl medium. To verify the specificity of hIL-10, the mAB 3F9 is included as an antagonist in some experiments.

Cell surface antigen expression

Cell surface expression of CD32/16 (Fcg II/III receptor) is analyzed, e.g., as described by Ho, et al. (1995) Mol. Cell. Biol. 15:5043-5053. Briefly, cells grown in medium containing mIL-3, various concentrations of hIL-10 or both cytokines, are washed once with FACS buffer (HBSS, 3% FCS, 0.02% sodium azide), and then incubated with FITC-conjugated anti-murine CD16/32 mAb (Pharmingen, San Diego CA) or isotype control for 30 min on ice. Cells are washed twice with FACS buffer and analyzed with a FACScan (Becton Dickinson).

Electrophoretic mobility shift assay

2-3 x 10^6 cells (HepG2 transfected the recombinant variants of IL-10RA and control) are washed and treated with various doses (0.01-100 ng) of hIL-10 or medium for various times (7 min - 24 hours). Whole cell extracts are prepared, e.g., by solubilizing cells in whole extract buffer (0.1 % Triton X-100, 10 mM HEPES, pH 7.9, 2 mM EDTA, 1 mM EGTA, 400 mM KCl, 1 mM DTT, and 10% glycerol with 1 mM orthovanadate, 0.5 mM PMSF, and 5 µg/ml each of pepstatin, leupeptin, and aprotinin) on ice for 30 min, which is

typically further cleared from insoluble material by centrifugation (10 000 x g) for 5 min at 4° C. Protein concentration of extracts is measured, e.g., by using the BCA-assay (Pierce) and 40 µg of cellular extract is incubated with, e.g., 25,000 cpm of an 32P-end labeled double stranded oligonucleotide probe based on 18 bps of the GRR of the FcgR1 gene (top strand (SEQ ID NO: 8): 5'-ATGTATTTCCCAGAAA-3'; bottom strand (SEQ ID NO: 9): 5'-CTTTTCTGGGAAATA-3') (Weber-Nordt, et al. (1996) J. Biol. Chem. 271:27954-27961) and the high affinity cis inducible element SIEm67: top strand (SEQ ID NO: 10): 5'-GATCTGATTACGGGAAATG-3' bottom strand (SEQ ID NO: 11): 5'-GATCCATTTCCCGTAATCA-3' (Sadowski, et al. (1993) Science 261:1739-1744). The incubation is performed, e.g., for 20 min at room temperature in 15 µl binding buffer (20 mM Hepes, pH 7.9, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, and 4% w/v Ficoll). The reaction mixture is separated, e.g., by electrophoresis on a 6% polyacrylamide gel in 0.25 x TBE for 3.5 h at 150 V. The gel is dried and analyzed by autoradiography. Specificity of the DNA binding complex is determined by incubating with a 100-fold excess of unlabeled GRR or SIEm67. To determine the identity of transcription factors present in the DNA binding complexes, supershift experiments are performed, e.g., by incubating extracts with antibodies specific for STAT-1 (anti-ISGF-3, Transduction Laboratories) or STAT-3 (C-20, Santa Cruz, Biotechnology) for 1 h at 4° C before the addition of the radiolabeled probes.

Phosphotyrosine specific Western blot analysis

Transfectants of HepG2 are treated with various doses of rhIL-10 (0.01-100 ng/ml) for various times (7 min to 24 hours) and lysed in a buffer containing, e.g., 50 mM Tris, pH 7.4, 150 mM NaCl, 30 mM NaPPi, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, 0.5 mM PMSF, 1 mM sodium vanadate, and 5 µg/ml each of pepstatin, leupeptin, and aprotinin. After 15 min incubation on ice, the extracts are cleared, e.g., by centrifugation (at 15,000 rpm, 4° C for 8 min) and concentration of proteins determined, e.g., by the BCA-assay (Pierce). Equal amounts of proteins are resolved by 10% SDS/PAGE and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). Membranes are probed with specific anti-phosphotyrosine Abs against STAT1 [Phospho-Stat1(Tyr701)], STAT3 [Phospho-STAT3(Tyr705), and anti-phosphoserine [Phospho-Stat3

(Ser727)] and then developed, e.g., using the enhanced chemiluminiscence system (ECL Amersham). The IL-10-mediated activation of STAT5 is analyzed in BAF-10R1/3, BAF-10R2/11 and control. Western blots are performed with a specific anti-phosphotyrosine antibody against STAT5 (Phospho-STAT5). Antibodies listed above are available, e.g., from New England Biolabs.

Reporter gene analysis

The CRP promoter fragment of 219 bp is excised from the CRP(219)-CAT construct and cloned, e.g., in the pGL3-basic vector (Promega) by standard procedures. The resultant reporter vector is sequenced and designated CRP(219)-Luc.

Transfectants of HepG2 are cultured, e.g., in six-wells, transiently (by the calcium phosphate method) transfected with 4 µg of a 1:1 mixture of the reporter gene construct pCRP(219)-luc and as an internal normalization marker pSV-beta-galactosidase (Promega). 24 h after transfection, the subcultures are either untreated or treated with various doses of hIL-10 for further 24 hours. 5 x 10^6 cells of each BAF-10R1/3, BAF-10R2/11, and parental Ba/F3 are transiently transfected, e.g., by electroporation with 10 µg of a 1:1 mixture of pXM-PRL(105) and pSV-beta-galactosidase vector. Subcultures are treated with various doses of hIL-10 for a period of 48 h. After treatment cells are lysed with Promega reporter lysis buffer and luciferase determined by using the Luciferase assay system (Promega) with a luminometer (Berthold). To account for differences in transfection efficiency, the activity of beta-galactosidase is determined on luminometer by using the Galacto-Star assay system (Tropix).

TNF-inhibition studies

Peripheral venous blood, anticoagulated with heparin, is drawn from 15 individuals with different IL-10RA genotypes and zygosity. Monocytes are isolated, e.g., by Ficoll-Hypaque density centrifugation and adherence, and then suspended at a density of 1 x 10^6 cells/ml in RPMI 1640 medium (GIBCO-BRL), which is supplemented with 10% heat-inactivated FCS, and glutamine. Aliquots of (0.2 ml) in microtiter wells of this PBMC suspension are left untreated, or treated either with 1 µg/ml LPS alone or together with various doses of hIL-10 (0.1-100 ng/ml) for 24 h. TNF-alpha secreted in the cell supernatants is assayed.

EXAMPLE 2: Natural Polymorphisms

Targeted disruption of the interleukin-10 (IL-10) gene in mice causes chronic intestinal inflammation which is susceptible to therapy with recombinant IL-10. As a consequence, rhIL-10 was studied for treatment of human inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis. A minority of patients showed a clinical improvement after administration of this cytokine. Nevertheless, in certain patients rhIL-10 induced complete endoscopic healing of ulcerated mucosa. This was most obvious in Crohn's disease when compared to a 0% response in the placebo treated control patients.

By searching for mutations in genes, which are involved in the IL-10 pathway and which could account for the favorable effect of rHuIL-10 in this distinct subgroup, a new mutation was identified in the cytoplasmic domain of the IL-10 receptor alpha chain. Additional polymorphic analysis identified a second site of variation.

IL-10RA genotype and response to rhIL-10

Seven Crohn's disease patients who had been treated with rhIL-10 (5-20 µg/kg per day for 4 weeks, sc) and had shown endoscopic improvement or healing (n=4) or no response (n=3) were chosen for analysis. PBMC's were isolated from each subject. After stimulation of PBMC with LPS, total RNA was extracted and subjected to cDNA synthesis. The entire coding sequence of the IL-10RA mRNA (=1737 bp) was sequenced after reverse transcription and rapid amplification of cDNA ends (5'-RACE) followed by PCR amplification of four overlapping segments on an automated sequencer (ABI 373, Perkin Elmer). In 8 out of 11 individuals a G to A transition at position 1051 was detected causing a substitution of glycine 351 to arginine (IL-10RA G351R). The data has also been confirmed by sequencing genomic DNA. The mutation is located in the cytoplasmic domain upstream from the tyrosine residues, Y446 and Y496 in an area (Aa 282-389) that, when deleted in mice (D282-389), leads to increased cell proliferation (Ho, et al., 1995). Interestingly, only Crohn's disease patients with this mutation (heterozygote or homozygote) showed a therapeutic response to rHuIL-10.

Two patients possessing the "standard" IL-10 receptor se-

quence only were not responsive to the administration of IL-10; three patients who were heterozygous with the described polymorphic G351R variation and "standard" were responsive, while one heterozygous patient was not responsive; and the one patient who exhibited homozygous variation was responsive to ligand administration.

Bi-directional PCR Amplification of Specific Alleles

For rapid detection of this mutation in larger cohorts, an allele specific PCR diagnosis was established, designated Bi-directional PCR Amplification of Specific Alleles (BiPASA). This method allowed distinguishing between the presence or absence and zygosity of the different IL-10RA genotypes in genomic DNA. The accuracy of this method was verified by a blinded comparison of BiPASA with cycle sequencing in 50 DNA samples. Both methods showed 100% identical results.

Detection of the different IL-10RA genotypes and zygosity by bi-directional PCR amplification of specific alleles (Bi-PASA):

To determine the different genotypes in the human IL-10RA gene two genetic test methods were developed. For each genetic variation in the coding sequence of the human interleukin-10 receptor alpha chain (IL-10RA) a separate test method was developed to detect the single-base changes and simultaneously distinguish between homozygotes and heterozygotes in one PCR reaction by utilizing a specific primer design and balanced concentration of primers with appropriate cycling conditions (Liu et al., 1997b)

The polymorphisms in the IL-10RA gene are typed by bi-directional PCR amplification of specific alleles (Bi-PASA) using 1-200 ng genomic DNA as template in each reaction. For each detection, four different primers (two outer primers designated as P and Q as well as 2 inner primer designated as A and B) were used in a single tube. PCR was performed using e.g. Taq DNA Polymerase (Promega) or AmpliTaq Gold (Applied Biosystems) with the buffers supplied from the manufacturer. The method can be easily adopted for high throughput population based analysis.

As an example Bi-Pasa can be performed under following cycling conditions using a thermal cycler (e.g. MJ Research; PTC 200):

Initial denaturation at 95 degree for 5 min
40 cycles with:
Denaturation: 95 degree for 45 sec
Primer annealing at 58-60 degree for 30 sec
Primer extension at 72 degree for 45 sec
Followed by a final extension at 72 degree for 7 min

PCR products are separated by size using standard agarose or polyacrylamide gel electrophoresis and visualized by specific staining such ethidium bromide or by other methods, which are well known in art. The size and number of PCR products represents genotype of the IL-10RA of the analyzed DNA sample.

Figure 1. Separated PCR products represent the IL-10 receptor alpha genotypes

Size of PCR fragments Bi-PASA of IL-10RA-G351R:
Control fragment IL-10RPQ-351: 515 bp;
Wildtype fragment IL-10RAQ-351: 318 bp
Variant fragment IL-10RPB-351: 248 bp

Size of PCR fragments for Bi-PASA of IL-10RA-S159G
Control fragment IL-10RPQ-159: 464 bp
Wildtype fragment IL-10RAQ-159: 337 bp
Variant fragment IL-10RPB-159: 183 bp

Detection of the IL-10RA genetic variant IL10RA-S159G: the corresponding A-G substitution at nucleotide 536 is numbered according to the published IL-10RA cDNA sequence (Genbank entry: U00672 or UniGene: Hs.327).

Primer sequences for Bi-PASA of IL-10RA-S159G
Outer primers:
IL-10RP-159: 5´- TCAGCCCTCAAGTCTCATGGTATTC-3´ (Seq.ID No.12)
IL-10RQ-159: 5´- TTGCTTCATCTACAAGGGCTCTGG-3´ (Seq.ID No.13)

Allele specific inner primers:
IL-10RA-159: 5'-GGGCGGGGCGGCRAATGACACATATGAAA-3' (Seq.ID No.14)
IL-10RB-159: 5´-GGGGCGGGGCGAAGTGACTGAAGATGCC-3´ (Seq.ID No.15)

Note: Primer IL-10RA-159 is specially designed with R (R= A+G) to avoid interference with the G->A substitution found at nucleotide 520 in the IL-10RA cDNA (Tanaka et al., 1997).

Detection of IL-10RA genetic variant IL10RA-G351R:
The corresponding G-A substitution at nucleotide 1112 is numbered according to the published IL-10RA cDNA sequence.

Primer sequences for BiPASA of IL-10RA-G351R
Outer primers:
IL-10RP-351: 5´-CCCTTCATCTTCATCAGCCAGCGTC-3´(Seq.ID No.16);
IL-10RQ-351: 5´-CTCAGGTAACCCTGGAATGCCACAG-3´(Seq.ID No.17);

Allele specific inner primers:
IL-10RA-351: 5´-GCCGGGGGGGAGAACGCTGGGAAACG-3´(Seq.ID No.18);
IL-10RB-351: 5´-GGGGGCGGGCCACAGGGGGCTCCCT-3'(Seq.ID No.19);

PCR primers suitable for use in this aspect of the invention are not limited to SEQ IDs: 12-19 but encompass all pairs or sets of PCR primers suitable for selective amplification of the region of an IL-10RA gene of an animal (preferably a human), or for amplification of that part of this region in which the polymorphism of the invention is located.

Variation 1 (IL-10RA-S159G):

An A to G substitution in the coding sequence of the original human IL-10RA cDNA sequence at nucleotide 536 (Genbank entry: U00672 or UniGene Hs.327) causing an amino acid conversion from serine to glycine at AA 159 in the extracellular domain of the interleukin-10 receptor alpha chain precursor (578 aa, Swissprot: locus I10R_HUMAN, Accession: Q13651).

cDNA Sequence IL-10RA (Seq.ID No.20):
521-AATGACACAT ATGAAAGCAT CTTCAGTCAC TTCCGAGAGT-561
cDNA Sequence IL-10RA-S159G (Seq.ID No.21):
521-AATGACACAT ATGAAGGCAT CTTCAGTCAC TTCCGAGAGT-561

Protein Sequence IL-10RA: 153-ANDTYESIFSHFREY-167
(Seq.ID No.22)
Protein Sequence IL-10RA-S159G: 153-ANDTYEGIFSHFREY-167

(Seq.ID No.23)

Variation 2 (IL-10RA-G351R):

An G to A substitution in the coding sequence of the human IL-10RA cDNA sequence at nucleotide 1112 (Genbank entry: U00672 or UniGene Hs.327) causing an amino acid conversion from glycine to arginine at AA 351 in the intercellular domain of the interleukin-10 receptor alpha chain precursor (578 aa, Swissprot: locus I10R_HUMAN, Accession: Q13651).

cDNA Sequence IL-10RA:
1101-CGCTGGGAAA CGGGGAGCCC CCTGTGCTGG-1130 (Seq.ID No.24)
cDNA Sequence IL-10RA-G351R:
1101-CGCTGGGAAA CAGGGAGCCC CCTGTGCTGG-1130 (Seq.ID No.25)

Protein sequence IL-10RA: 341-HPQADRTLGNGEPPVLGDSC-360 (Seq.ID No.26)
Protein sequence IL-10RA-G351R: 341-HPQADRTLGNREPPVLGDSC-360 (Seq.ID No.27)

Frequency of IL-10RA G351R in the normal population:

One hundred twenty healthy individuals were recruited out of hospital employees and patients with gallstones, inguinal hernia, or routine screening for thyroid function. The mean age was 45.4 years (15-86 years) and 65% were female. BiPASA analysis of DNA samples showed that 11/120 (9.2%) were homozygous for the IL-10RA G351R, 46/120 (38.3%) were heterozygous, and 63/120 (52.5%) were homozygous for the IL-10RA wildtype. From the 240 chromosomes under investigation, the frequency of IL-10RA G351R was 29% vs. 71% wildtype. The "standard" allele is more frequent by a factor 2.43. IL-10RA G351R has to be regarded as single nucleotide polymorphism (SNP); other SNPs should exist and elucidation of their properties and functions would be well within the capabilities of the practitioner.

Frequency of Il-10RA S159G in the normal population:

BiPASA analysis of 99 control subjects showed that 1 (1%) sample was homozygous for IL-10RA-S159G, 24 (24%) were heterozy-

gous, and 74 (75%) were homozygous for the wildtype. From 198 chromosomes under investigation, the frequency of IL-10RA-S159G was 13.13% vs. 86.87% wildtype. The standard allele is more frequent by the factor 6.62. The IL-10RA-S159G has to be regarded as a SNP. The finding that IL-10RA-S159G changes IL-10 binding is supported by Reineke et al., 1998; Hoover et al., 1999; Zdanov et al., 1996; Zdanov et al., 1997; Walter and Nagabhushan, 1995.

EXAMPLE 3: Disease correlations

For the study of the biological significance of the IL-10 G351R, the following issues may be addressed.

Susceptibility to inflammatory bowel disease

Unrelated patients with Crohn's disease and patients with ulcerative colitis may be prospectively analyzed for their IL-10RA genotypes using BiPASA. Recruitment of patient's parents may be done by mail. DNA may be sampled, e.g., peripheral blood may be drawn from patients and their parents. Detailed analysis can be recorded, e.g., the location pattern of ulcerative colitis patients, the Vienna Classification of Crohn's disease, and the level of pANCA and pASCA in serum can be applied for subgroup analysis. The transmission disequilibrium test and chi square test can be used to evaluate the frequency of allelic transmission to affected offspring. Spielman and Ewens (1996) Am. J. Hum. Genet. 56:11-14.

Susceptibility to autoimmune disease

DNA samples from patients with SLE and rheumatoid arthritis can be evaluated. A DNA bank from such patients might be evaluated, alternatively new patients may be recruited. For example, patients may be selected according to whether they meet at least 4 criteria of the American College of Rheumatology for SLE. Samples can be grouped according to various criteria, e.g., organ involvement, damage index, activity index, and serologic profile (anti-DNA antibodies, ENA, complement deficiency, HLA-DR). Some samples of DNA from first degree relatives may also be analyzed.

Correlations to previous studies on IL-10 promoter polymorphisms in patients may be evaluated. Data may be compared to control subjects, e.g., from the same ethnicity.

Similar studies to determine correlation of medical condi-

tion with receptor polymorphism may be applied, e.g., to EBV associated lymphoproliferative disorders (post-transplant lymphoproliferative disease, Burkitt's lymphoma); chronic lymphatic leukemia; chronic graft-versus-host diseases; septic shock/meningococcal infection and patient survival; Type I diabetes; atopic dermatitis; IL-10 in Hepatitis C; autoimmune hepatitis; and others. See, e.g., Berkow (ed.) The Merck Manual of Diagnosis and Therapy, Merck & Co., Rahway, N.J.; Thorn, et al. Harrison's Principles of Internal Medicine McGraw-Hill, NY.

EXAMPLE 4: Mechanistic studies

To further elucidate functional differences between standard and IL-10RA polymorphic variants, additional in vitro studies are possible.

Induction of proliferation and surface antigen expression

Recent studies have shown that recombinant murine IL-10RA expressed in the IL-3-dependent murine pro-B-cell line Ba/F3 can transduce a proliferative signal in response to IL-10. Ho, et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:11267-11271; and Ho, et al. (1995) Mol. Cell. Biol. 15:5043-5053. IL-10-induced growth leads to differentiation which becomes evident by formation of large clusters of cells and by induction or enhancement of several surface antigens, including CD32/16, CD2, LECAM-1, and heat stable antigen. Ba/F3 transfectants expressing membrane-proximal deletion mutants of mIL-10RA (D282-389, D282-414, and D282-458) displayed a 30 to 100 fold higher sensitivity to IL-10.

In addition to studies in the murine pro-B-cell line Ba/F3, the recombinant human IL-10R have been expressed in a human erythroleukemia cell line TF-1. HIL-10 can also induce a short term proliferative response in human TF-1 cells expressing recombinant hIL-10RA. Liu, et al. (1997) J. Immunol. 158:604-613. As the functional characterization of mIL-10RA has already been done in Ba/F3 (Ho, et al., 1995), this hIL-10RA polymorphic variant will be first studied in this cell line. Additional proliferation studies may be performed in human TF-1 cells to exclude human/mouse species differences in the engagement between IL-10RA and IL-10RB.

Differences in IL-10RA expression, ligand binding affinities and internalization between the "standard" (wild type) and poly-

morphic IL-10RA will be determined by Scatchard plot analysis. Studies with EBV immortalized lymphoblastoid cells from individuals with different IL-10RA genotype and zygosity may also be subjected to similar analysis.

Signaling through the JAK-STAT pathway

The mutation IL-10R G351R lies within the membrane-proximal region, which suggests a focus on the IL-10RA variants on the IL-10 mediated activation of the different STAT factors and their potential to activate transcription of reporter genes. In a recent article, receptor signaling of IL-10 was studied in comparison with IL-6 via the induction of transcription through IL-6 responsive elements in the HepG2 cell line stably transfected with hIL-10RA. Lai, et al. (1996) J. Biol. Chem. 271:13968-13975. Different IL-6 responsive reporter constructs have been tested and the IL-10-mediated induction in the pCRP(219)-CAT (219-bp of the human C-reactive protein promoter, Ganter, et al. (1989) EMBO J. 8: 3773-3779) exceeded the IL-6 effect by several fold. The capability of IL-10RA variants to activate the 219-bp CRP promoter construct can be analyzed, e.g., by a luciferase reporter gene construct. The IL-10-mediated activation of STAT5 can be examined in transfectants of Ba/F3 using a luciferase reporter gene construct which is under the control of the b-casein promoter as described previously. See Wehinger, et al. (1996) FEBS Lett. 394:365-370.

Inhibition of LPS-stimulated TNF-alpha production

Stimulation of monocytes with bacterial lipopolysaccharides (LPS) induces expression of multiple cytokines, including TNF-alpha, IL-1b, and IL-6. Furthermore, IL-10 feedback inhibits expression of TNF-alpha, IL-1b, and IL-6, thus providing an efficient autocrine mechanism for controlling proinflammatory cytokine production in monocytes. See, e.g., de Waal Malefyt, et al. (1991) J. Exp. Med. 174:1209-20; Wang, et al.(1994) J. Immunol. 153:811-816; Donnelly, et al. (1995) J. Immunol. 155:1420-1427; and Aste-Amezaga, et al. (1998) J. Immunol. 160:5936-5944. Treatment of monocytes in vitro with rhIL-10 starting 20 hours before to 1 hours after exposure to LPS have been demonstrated to inhibit the secretion of TNF-alpha.

To study the sensitivity between the different IL-10RA geno-

types, PBMCs will be isolated from individuals with different IL-10RA genotypes and treated with various concentrations of rhIL-10 and LPS. Accumulation of TNF-alpha in the supernatant will be determined and the inhibition of LPS-induced TNF-alpha production in response to various doses of rhIL-10 will be plotted in percent of control (no rhIL-10).

Detection of the different IL-10RA genotypes and zygosity by bidirectional PCR amplification of specific alleles (Bi-PASA):

The exploration of the relationship between genome variation (SNPs) and complex phenotypes (common disease) is of clinical importance. A principal strategy is based upon large-scale analysis of intragenic SNPs in common disorders, resulting in the development of a number of internationally recognized tools and research strategies. SNPs, which are small alterations in DNA that vary from person to person, are expected to serve as genetic markers of predisposition to disease and may pave the way for the development of novel diagnostics and drugs tailored to an individual's genetic profile.

To determine the different genotypes in the human IL-10RA gene two genetic test methods were developed. For each genetic variation in the coding sequence of the human interleukin-10 receptor alpha chain (IL-10RA) a separate test method was developed to detect the single-base changes and simultaneously distinguish between homozygotes and heterozygotes in one PCR reaction by utilizing a specific primer design and balanced concentration of primers with appropriate cycling conditions (Overlapping PCR for bidirectional PCR amplification of specific alleles: a rapid one-tube method for simultaneously differentiating homozygotes and heterozygotes. Liu-Q; Thorland-EC; Heit-JA; Sommer-SS Genome-Res. 1997 Apr; 7(4): 389-98).

Detection of IL-10RA genetic variant IL10RA-G351R the corresponding G-A substitution at nucleotide number 1112 according to the published IL-10RA cDNA sequence (Genbank entry :U00672 or UniGene: Hs.327).

Primer sequences for genotyping of IL-10RA-G351R, artificial GC-clamps are underlined:

outer primers:

IL-10RP-351: 5´-CCCTTCATCTTCATCAGCCAGCGTC-3´ (Seq.ID No.16);
IL-10RQ-351: 5´-CTCAGGTAACCCTGGAATGCCACAG-3´ (Seq.ID No.17);

allele specific inner primers:
IL-10RA-351: 5´-GCCGGGGGGGAGAACGCTGGGAAACG-3´ (Seq.ID No.18);
IL-10RB-351: 5´-GGGGGCGGGCCACAGGGGGCTCCCT-3' (Seq.ID No.19);

PCR primers suitable for use in this aspect of the invention are not limited to SEQ IDs (specify above listed primers) but encompass all pairs or sets of PCR primers suitable for selective amplification of the region of an IL-10RA gene of an animal (preferably a human), or for amplification of that part of this region in which the polymorphism of the invention is located.

Detection of IL-10RA genetic variants IL10RA-S159G the corresponding A-G substitution at nucleotide number 536 according to the published IL-10RA cDNA sequence:

outer primers:
IL-10RP-159: 5´- TCAGCCCTCAAGTCTCATGGTATTC-3´ (Seq.ID No.12)
IL-10RQ-159: 5´- TTGCTTCATCTACAAGGGCTCTGG-3´ (Seq.ID No.13)

allele specifc inner primers:
IL-10RA-159: 5´- GggcggggcgGCRAATGACACATATGAAA (Seq.ID No.14)
(Primer has adapted to avoid interference with the already published G->A substitution at nucleotide 520)
Il-10RB-159: 5´-GGGGCGGGGCGAAGTGACTGAAGATGCC-3´ (Seq.ID No.15)

Note : Primer IL-10RA-159 is specially designed with R (R= A+G) to avoid an interference with the G->A substitution found at nucleotide 520 in the IL-10RA cDNA (Detection of polymorphisms within the human IL10 receptor cDNA gene sequence by RT-PCR RFLP. Tanaka Y, Nakashima H, Otsuka T, Nemoto Y, Niiro H, Yamaoka K, Ogami E, Arinobu Y, Tachida H, Imamura T, Niho Y. Immunogenetics. 1997;46(5):439-41).

Variation 1: An A to G substitution in the coding sequence of the original human IL-10RA cDNA sequence at nucleotide 536 (Genbank entry :U00672 or UniGene Hs.327) causing an amino acid conversion from serine to glycine at AA 159 in the extracellular

domain of the interleukin-10 receptor alpha chain precursor (578 aa, Swissprot: locus I10R_HUMAN, Acession: Q13651).

cDNA Sequence IL-10RA:
521-AATGACACATATGAAAGCATCTTCAGTCACTTCCGAGAGT-561 (Seq.ID No.20)

cDNA Sequence IL-10RA-S159G:
521-AATGACACATATGAAGGCATCTTCAGTCACTTCCGAGAGT-561 (Seq.ID No.21)

Protein Sequence IL-10RA: AA 159 from IL-10RA: ANDTYESIFSHFREY (Seq.ID No.22) change to ANDTYEGIFSHFREY (Seq.ID No.23)

Variation 2: An G to A substitution in the coding sequence of the human IL-10RA cDNA sequence at nucleotide 1112 (Genbank entry :U00672 or UniGene Hs.327) causing an amino acid conversion from glycine to arginine at aa 351 in the intercellular domain of the interleukin-10 receptor alpha chain precursor (578 aa, Swissprot: locus I10R_HUMAN, Acession: Q13651).

cDNA Sequence IL-10RA:
nt1101-CGCTGGGAAACGGGGAGCCCCCTGTGCTGG-nt1130 (Seq.ID No.24)
cDNA Sequence IL-10RA-G351R:
nt1101-CGCTGGGAAACAGGGAGCCCCCTGTGCTGG-nt1130 (Seq.ID No.25)

Protein sequence IL-10RA: HPQADRTLGNGEPPVLGDSC (Seq.ID No.26)
Protein sequence IL-10RA-G351R: HPQADRTLGNREPPVLGDSC (Seq.ID No.27)

PCR test methods for genotyping IL-10RA variants:

The polymorphisms in the IL-10RA gene were typed by PCR using 1ng - 200ng genomic DNA as template in each reaction e.g. in a total volume of 25 µl. For each detection method four different primers two outer primers designated as P and Q as well as 2 inner primer designated as A and B. PCR was performed using e.g. Taq DNA Polymerase (Promega) or AmpliTaq Gold (Applied Biosystems) with the buffers supplied from the manufacturer. Optimization strategies for multiplex PCR reactions are well known in art can be gener-

ally obtained by varying the ionic strength of the reaction buffer (increase or decrease of K+ concentration), concentration of Mg2+, dNTPs, primer design and the concentration of the individual primers and the use of cosolvents such DMSO, trehalose, glycerol or betaine (PCR-books).

A bidirectional PCR amplification of specific alleles (Bi-Pasa) was developed to screen genotypes of IL-10RA by using genomic DNA as template. The method can be easily adopted for high throughput population based analysis.

As an example Bi-Pasa can perfomed under following cycling conditions using a thermal cycler (e.g. MJ Research; PTC 200 as well as other commercialy available thermal cyclers):

initial denaturation at 95 degree for 5 min
40 cycles with:
denaturation: 95 degree for 45 sec
primer anealing at 58-60 degree for 30 sec
primer extension at 72 degree for 45 sec
followed by a final extension at 72 degree for 7 min

PCR products are separated by size using standard agarose or polyacrylamid gel electrophoresis and visualized by specific stainings such ethidium bromide or by other methods which are well known in art. The size and number of PCR products represents genotype of the IL-10RA of the analyzed DNA sample.

Figure 1. Separated PCR products represents the IL-10 receptor alpha genotypes

Size of PCR fragments Bi-Pasa of IL-10RA-G351R:
genomic control fragment 515 bp;
wildtype fragment IL-10RAQ 318 bp
variant fragment IL-10RPB: 248 bp

Size of PCR fragments for Bi-Pasa for detecing IL-10RA-S159G
genomic control fragment: 464 bp
wildtype fragment IL-10RAQ 337 bp
variant fragment IL-10RPB 183 bp

Frequency of Il-10RA S159G in the normal population:

BiPASA analysis of DNA samples showed that 1/99 (1.0 %) were homozygote for IL-10RA S159G , 24/99 were heterozygous, and 74/99 were homozygous in the wildtype form. From 198 chromosomes under investigation the the frequency of IL-10RA S159G was 13.13% vs. 86.87% wildtype. The standard allele is more frequent by the factor 6.62.

Sequence ID No.38 (genomic fragment to detect human IL-10RA-S159G with BiPASA)
464
DNA
mammal

TCAGCCCTCA AGTCTCATGG TATTCCCCCC CACCCCAACT CCATTTAGTG ACTCTGACAG 60
TTGGCAGTGT GAACCTAGAG ATCCACAATG GCTTCATCCT CGGGAAGATT CAGCTACCCA 120
GGCCCAAGAT GGCCCCCGCG AATGACACAT ATGAAAGCAT CTTCAGTCAC TTCCGAGAGT 180
ATGAGATTGC CATTCGCAAG GTGCCGGGAA ACTTCACGGT ATGGGGTTCC CCAAGGCCCC 240
AGGGCCAGAA CTCCCTTGGC TTCCCTGTCC CCTGGGCTGG AAGCACCCTT GTGTGCCATT 300
GGGAACTTTG CTTATGGACT AAAGGGAGGG TCTGGGATGG TGGGTGGGCA GAGGAGGGAG 360
TAGAAACCAC CTCAGCCCTC AGAGCTATGC TCTTGTGAGA TGCTCCTCAA AGGAATTGCC 420
TACTAGGGCA ATTGGGCACC CCAGAGCCCT TGTAGATGAA GCAA 464

Sequence ID No.39 (genomic fragment to detect human IL-10RA-G351R; " NOTE.: NO DIFFERENCE BETWEEN Cdna AND DNA in this fragment)
515
DNA
mammal

CCCTTCATCT TCATCAGCCA GCGTCCCTCC CCAGAGACCC AAGACACCAT CCACCCGCTT 60
GATGAGGAGG CCTTTTTGAA GGTGTCCCCA GAGCTGAAGA ACTTGGACCT GCACGGCAGC 120
ACAGACAGTG GCTTTGGCAG CACCAAGCCA TCCCTGCAGA CTGAAGAGCC CCAGTTCCTC 180
CTCCCTGACC CTCACCCCCA GGCTGACAGA ACGCTGGGAA ACGGGGAGCC CCCTGTGCTG 240
GGGGACAGCT GCAGTAGTGG CAGCAGCAAT AGCACAGACA GCGGGATCTG CCTGCAGGAG 300
CCCAGCCTGA GCCCCAGCAC AGGGCCCACC TGGGAGCAAC AGGTGGGGAG CAACAGCAGG 360
GGCCAGGATG ACAGTGGCAT TGACTTAGTT CAAAACTCTG AGGGCCGGGC TGGGGACACA 420
CAGGGTGGCT CGGCCTTGGG CCACCACAGT CCCCCGGAGC CTGAGGTGCC TGGGGAAGAA 480
GACCCAGCTG CTGTGGCATT CCAGGGTTAC CTGAG 515

Analysis of the splice site mutation

Peripheral blood mononuclear cells (PBMCs) from seven pa-

tients with Crohns disease and four healthy donors were isolated from EDTA-whole blood using Ficoll-Paque Plus separation gradient (Pharmacia Biotech) following manufacturer's guidelines. Total RNA from PBMCs was isolated using TRI-Reagent® (Molecular Research Center Inc.; Cincinnati, OH) according to manufacturer's instructions. Utilizing the 5´-Race system for rapid amplification of cDNA ends (Life Technologies Inc, Rockville,MD), one microgram of each RNA were reverse transcribed using the using SuperScript II and oligodT(18) primer according to the manufacturers instructions. After purification and precipitation the cDNA-reaction was diluted in 50µl TE buffer. To analyze entire coding region of IL-10RA mRNA four different overlapping fragments were generated by PCR in separate reaction tubes using sequence specific primers. For amplification of the 5´-untranslated region of the IL-10RA the 5´ Race abridged anchored primer from the 5´-Race system for rapid amplification of cDNA ends was used together with a sequence specific primer (see fragment 1). Aliquots of 5µl from the diluted cDNA reaction were amplified by PCR using 1x PCR buffer, Mg2+and dNTP (all reagents from Promega, Madison, WI) and 10 pmol of each primer in a total volume of 50µl. To increase the yield and fidelity of PCR a 3:1 mixture of Taq-DNA Polymerase (Promega, Madison, WI) and Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) was used instead of Taq DNA polymerase alone as recommended by the instructions of the manufaturer.

cycling conditions were used for PCR:
initial denaturation at 95 degree for 45 sec
35 cycles with:
denaturation: 95 degree for 45 sec
primer anealing at 58 degree for 30 sec
primer extension at 72 degree for 45 sec
followed by a final extension at 72 degree for 7 min

Following primers were used for PCR amplification and cycle sequencing reaction.

Fragment 1:
5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3´ (Seq.ID No.28);
5´-GGCAATCTCATACTCTCGGAAG-3 (Seq.ID No.29)

Fragment 2:
5´-TCATCCTCGGGAAGATTCAG-3´ (Seq.ID No.30);
5´-GTCCAAGTTCTTCAGCTCTGG-3´ (Seq.ID No.31),
Fragment 3:
5´-TTCATCAGCCAGCGTCCCTC-3´ (Seq.ID No.32);
5´-TGGTCTGCCTCAGGTAACCC-3´ (Seq.ID No.33);
Fragment 4:
5´-CAGGGTTACCTGAGGCAGAC-3´ (Seq.ID No.34);
5´-TGCCTCGTGCCTAACTTCTG-3´ (Seq.ID No.35);

RT-PCR products were analyzed by standard agarose gel electrophoresis. Beside the expected PCR products an additional truncated PCR product appeared in fragment 1 in one out of ten analyzed. The truncated form was isolated from the agarose gel and subjected to cycle sequencing and analyzed on a automatic sequencer (ABI-Prism 310, Applied Biosystems, Foster City, CA). Additionally the coding sequence of the truncated IL-10RA cDNA of this individual was amplified with IL-10R-EcoRV (Seq ID No: 5) and IL-10R-EcoRI (Seq ID NO: 6) subloned in the expression vector pIRES-puro und further analyzed by sequencing. Sequence analysis showed that 179 bp of the IL-10RA coding sequence were spliced out in the truncated form of human IL-10RA. This phenomena was further characterized by analyzing the genomic intron-exon boundries based on the draft of the human genome sequence. The genomic localization of the human IL-10RA has already been been mapped on chromsome 11q23.3. Taniyama, T.; et al (1995) Hum. Genet. 95: 99-101. Search on public genomic DNA databases such as Megablast at National Center of Biotechnology Information showed that the 179 bp of the skipped exon has 100% homology on accession: AP000665 Homo sapiens genomic DNA, chromosome 11q, clone:CMB9-46G18. Based on these homology two primers were designed to amplify by PCR a region which covers the exon-intron boundries of the skipped exon sequence. The amplified PCR-product of 297 bp was purified with Qiaquick PCR purification kit (Qiagen, Hilden, Germany), and subjected to cycle sequencing reaction and analyzed on an automatic sequencer (ABI-Prism 310, Applied Biosystems, Foster City, CA). Sequence analysis of this region showed a G-to-A substitution in the acceptor splice site of the exon (GTGGATGAAG/GTGCTTTT change to GTGGATGAAA/GTGCTTTT) in the intron-exon boundary at position 255 of the genomic fragment of

IL-10RA. This single base substitution cause a splice site mutation which leads to an exon-skipping during the synthesis of IL-10RA mRNA. The mutation was found in a heterozygotic manner in one healthy donor. Further sequence analysis on 25 unrelated patients with Crohns disease in order to identify the causative mutations in affected individuals and to determine the prevalence of this splice site mutation were carried out. None of these patients were found to be carriers of the mutation. The predicted protein sequence of the truncated form of IL-10RA mRNA leads to only 94 amino acids. Thereby only the first 62 amino acids are processed in the same manner as in the wildtype followed by an altered protein sequence of 32 aminoacids and a stop codon. It can be assumed that an individual with an homozygotic form of this mutation lacks a functional IL-10 receptor complex and must have similar phenotype as can observed in IL-10 knock out mice. Kuhn, R., et al (1993). Cell 75: 263-74.

Primers to amplify and analyze the genomic exon-intorn boundary:

Intron-Exon-FW: 5´-GCCTCTTGCGTCTCCCTTAAAG-3´ (Seq.ID No.36);

Intron-Exon-RV: 5´-GGGACTTCAGAGCCATGTTCTAAG-3´ (Seq.ID No.37);

Sequence ID No.1 (human IL-10RA nucleic acid)

1737

DNA

homo sapiens

ATGCTGCCGT GCCTCGTAGT GCTGCTGGCG GCGCTCCTCA GCCTCCGTCT TGGCTCAGAC 60

GCTCATGGGA CAGAGCTGCC CAGCCCTCCG TCTGTGTGGT TTGAAGCAGA ATTTTTCCAC 120

CACATCCTCC ACTGGACACC CATCCCAAAT CAGTCTGAAA GTACCTGCTA TGAAGTGGCG 180

CTCCTGAGGT ATGGAATAGA GTCCTGGAAC TCCATCTCCA ACTGTAGCCA GACCCTGTCC 240

TATGACCTTA CCGCAGTGAC CTTGGACCTG TACCACAGCA ATGGCTACCG GGCCAGAGTG 300

CGGGCTGTGG ACGGCAGCCG GCACTCCAAC TGGACCGTCA CCAACACCCG CTTCTCTGTG 360

GATGAAGTGA CTCTGACAGT TGGCAGTGTG AACCTAGAGA TCCACAATGG CTTCATCCTC 420

GGGAAGATTC AGCTACCCAG GCCCAAGATG GCCCCCGCGA ATGACACATA TGAAAGCATC 480

TTCAGTCACT TCCGAGAGTA TGAGATTGCC ATTCGCAAGG TGCCGGGAAA CTTCACGTTC 540

ACACACAAGA AAGTAAAACA TGAAAACTTC AGCCTCCTAA CCTCTGGAGA AGTGGGAGAG 600

TTCTGTGTCC AGGTGAAACC ATCTGTCGCT TCCCGAAGTA ACAAGGGGAT GTGGTCTAAA 660

GAGGAGTGCA TCTCCCTCAC CAGGCAGTAT TTCACCGTGA CCAACGTCAT CATCTTCTTT 720

GCCTTTGTCC TGCTGCTCTC CGGAGCCCTC GCCTACTGCC TGGCCCTCCA GCTGTATGTG 780

CGGCGCCGAA AGAAGCTACC CAGTGTCCTG CTCTTCAAGA AGCCCAGCCC CTTCATCTTC 840
ATCAGCCAGC GTCCCTCCCC AGAGACCCAA GACACCATCC ACCCGCTTGA TGAGGAGGCC 900
TTTTTGAAGG TGTCCCCAGA GCTGAAGAAC TTGGACCTGC ACGGCAGCAC AGACAGTGGC 960
TTTGGCAGCA CCAAGCCATC CCTGCAGACT GAAGAGCCCC AGTTCCTCCT CCCTGACCCT 1020
CACCCCCAGG CTGACAGAAC GCTGGGAAAC GGGGAGCCCC CTGTGCTGGG GGACAGCTGC 1080
AGTAGTGGCA GCAGCAATAG CACAGACAGC GGGATCTGCC TGCAGGAGCC CAGCCTGAGC 1140
CCCAGCACAG GGCCCACCTG GGAGCAACAG GTGGGGAGCA ACAGCAGGGG CCAGGATGAC 1200
AGTGGCATTG ACTTAGTTCA AAACTCTGAG GGCCGGGCTG GGGACACACA GGGTGGCTCG 1260
GCCTTGGGCC ACCACAGTCC CCCGGAGCCT GAGGTGCCTG GGGAAGAAGA CCCAGCTGCT 1320
GTGGCATTCC AGGGTTACCT GAGGCAGACC AGATGTGCTG AAGAGAAGGC AACCAAGACA 1380
GGCTGCCTGG AGGAAGAATC GCCCTTGACA GATGGCCTTG GCCCCAAATT CGGGAGATGC 1440
CTGGTTGATG AGGCAGGCTT GCATCCACCA GCCCTGGCCA AGGGCTATTT GAAACAGGAT 1500
CCTCTAGAAA TGACTCTGGC TTCCTCAGGG GCCCCAACGG GACAGTGGAA CCAGCCCACT 1560
GAGGAATGGT CACTCCTGGC CTTGAGCAGC TGCAGTGACC TGGGAATATC TGACTGGAGC 1620
TTTGCCCATG ACCTTGCCCC TCTAGGCTGT GTGGCAGCCC CAGGTGGTCT CCTGGGCAGC 1680
TTTAACTCAG ACCTGGTCAC CCTGCCCCTC ATCTCTAGCC TGCAGTCAAG TGAGTGA 1737

Sequence ID No.2 (human IL-10RA amino acid)
578
PRT
homo sapiens

MLPCLVVLLA ALLSLRLGSD AHGTELPSPP SVWFEAEFFH HILHWTPIPN QSESTCYEVA 60
LLRYGIESWN SISNCSQTLS YDLTAVTLDL YHSNGYRARV RAVDGSRHSN WTVTNTRFSV 120
DEVTLTVGSV NLEIHNGFIL GKIQLPRPKM APANDTYESI FSHFREYEIA IRKVPGNFTF 180
THKKVKHENF SLLTSGEVGE FCVQVKPSVA SRSNKGMWSK EECISLTRQY FTVTNVIIFF 240
AFVLLLSGAL AYCLALQLYV RRRKKLPSVL LFKKPSPFIF ISQRPSPETQ DTIHPLDEEA 300
FLKVSPELKN LDLHGSTDSG FGSTKPSLQT EEPQFLLPDP HPQADRTLGN GEPPVLGDSC 360
SSGSSNSTDS GICLQEPSLS PSTGPTWEQQ VGSNSRGQDD SGIDLVQNSE GRAGDTQGGS 420
ALGHHSPPEP EVPGEEDPAA VAFQGYLRQT RCAEEKATKT GCLEEESPLT DGLGPKFGRC 480
LVDEAGLHPP ALAKGYLKQD PLEMTLASSG APTGQWNQPT EEWSLLALSS CSDLGISDWS 540
FAHDLAPLGC VAAPGGLLGS FNSDLVTLPL ISSLQSSE 578

Sequence ID No.3 (genomic fragment to analyze the splice site mutation)
297
DNA
mammal

GCCTCTTGCG TCTCCCTTAA AGGAGGTAGG ATTGAGCACA AGCTCGTTTC CAGTGCCTAA 60
CCTGGTATCT CCTCAGGTAT GGAATAGAGT CCTGGAACTC CATCTCCAAC TGTAGCCAGA 120
CCCTGTCCTA TGACCTTACC GCAGTGACCT TGGACCTGTA CCACAGCAAT GGCTACCGGG 180
CCAGAGTGCG GGCTGTGGAC GGCAGCCGGC ACTCCAACTG GACCGTCACC AACACCCGCT 240
TCTCTGTGGA TGAAGGTGCT TTTCCTCCCT TGACTTAGAA CATGGCTCTG AAGTCCC 297

Sequence ID No.4 (truncated human IL-10RA sequence)

DNA

1558

DNA

mammal

ATGCTGCCGT GCCTCGTAGT GCTGCTGGCG GCGCTCCTCA GCCTCCGTCT TGGCTCAGAC 60
GCTCATGGGA CAGAGCTGCC CAGCCCTCCG TCTGTGTGGT TTGAAGCAGA ATTTTTCCAC 120
CACATCCTCC ACTGGACACC CATCCCAAAT CAGTCTGAAA GTACCTGCTA TGAAGTGGCG 180
CTCCTGAGTG ACTCTGACAG TTGGCAGTGT GAACCTAGAG ATCCACAATG GCTTCATCCT 240
CGGGAAGATT CAGCTACCCA GGCCCAAGAT GGCCCCCGCG AATGACACAT ATGAAAGCAT 300
CTTCAGTCAC TTCCGAGAGT ATGAGATTGC CATTCGCAAG GTGCCGGGAA ACTTCACGTT 360
CACACACAAG AAAGTAAAAC ATGAAAACTT CAGCCTCCTA ACCTCTGGAG AAGTGGGAGA 420
GTTCTGTGTC CAGGTGAAAC CATCTGTCGC TTCCCGAAGT AACAAGGGGA TGTGGTCTAA 480
AGAGGAGTGC ATCTCCCTCA CCAGGCAGTA TTTCACCGTG ACCAACGTCA TCATCTTCTT 540
TGCCTTTGTC CTGCTGCTCT CCGGAGCCCT CGCCTACTGC CTGGCCCTCC AGCTGTATGT 600
GCGGCGCCGA AAGAAGCTAC CCAGTGTCCT GCTCTTCAAG AAGCCCAGCC CCTTCATCTT 660
CATCAGCCAG CGTCCCTCCC CAGAGACCCA AGACACCATC CACCCGCTTG ATGAGGAGGC 720
CTTTTTGAAG GTGTCCCCAG AGCTGAAGAA CTTGGACCTG CACGGCAGCA CAGACAGTGG 780
CTTTGGCAGC ACCAAGCCAT CCCTGCAGAC TGAAGAGCCC CAGTTCCTCC TCCCTGACCC 840
TCACCCCCAG GCTGACAGAA CGCTGGGAAA CGGGGAGCCC CCTGTGCTGG GGGACAGCTG 900
CAGTAGTGGC AGCAGCAATA GCACAGACAG CGGGATCTGC CTGCAGGAGC CCAGCCTGAG 960
CCCCAGCACA GGGCCCACCT GGGAGCAACA GGTGGGGAGC AACAGCAGGG GCCAGGATGA 1020
CAGTGGCATT GACTTAGTTC AAAACTCTGA GGGCCGGGCT GGGGACACAC AGGGTGGCTC 1080
GGCCTTGGGC CACCACAGTC CCCCGGAGCC TGAGGTGCCT GGGGAAGAAG ACCCAGCTGC 1140
TGTGGCATTC CAGGGTTACC TGAGGCAGAC CAGATGTGCT GAAGAGAAGG CAACCAAGAC 1200
AGGCTGCCTG GAGGAAGAAT CGCCCTTGAC AGATGGCCTT GGCCCCAAAT TCGGGAGATG 1260
CCTGGTTGAT GAGGCAGGCT TGCATCCACC AGCCCTGGCC AAGGGCTATT TGAAACAGGA 1320
TCCTCTAGAA ATGACTCTGG CTTCCTCAGG GGCCCCAACG GGACAGTGGA ACCAGCCCAC 1380
TGAGGAATGG TCACTCCTGG CCTTGAGCAG CTGCAGTGAC CTGGGAATAT CTGACTGGAG 1440
CTTTGCCCAT GACCTTGCCC CTCTAGGCTG TGTGGCAGCC CCAGGTGGTC TCCTGGGCAG 1500
CTTTAACTCA GACCTGGTCA CCCTGCCCCT CATCTCTAGC CTGCAGTCAA GTGAGTGA 1558

Sequence ID No.5 (truncated human IL-10RA protein)

94

PRI

mammal

MLPCLVVLLA ALLSLRLGSD AHGTELPSPP SVWFEAEFFH HILHWTPIPN QSESTCYEVA 60

LLSDSDSWQC EPRDPQWLHP REDSATQAQD GPRE 94

Sequence ID No.40 IL-10RA cDNA according to UniGene Cluster Hs.327 IL10RA and Genbank entry : U00672

3632

DNA

mammal (homo sapiens)

AAAGAGCTGG AGGCGCGCAG GCCGGCTCCG CTCCGGCCCC GGACGATGCG GCGCGCCCAG 60

GATGCTGCCG TGCCTCGTAG TGCTGCTGGC GGCGCTCCTC AGCCTCCGTC TTGGCTCAGA 120

CGCTCATGGG ACAGAGCTGC CCAGCCCTCC GTCTGTGTGG TTTGAAGCAG AATTTTTCCA 180

CCACATCCTC CACTGGACAC CCATCCCAAA TCAGTCTGAA AGTACCTGCT ATGAAGTGGC 240

GCTCCTGAGG TATGGAATAG AGTCCTGGAA CTCCATCTCC AACTGTAGCC AGACCCTGTC 300

CTATGACCTT ACCGCAGTGA CCTTGGACCT GTACCACAGC AATGGCTACC GGGCCAGAGT 360

GCGGGCTGTG GACGGCAGCC GGCACTCCAA CTGGACCGTC ACCAACACCC GCTTCTCTGT 420

GGATGAAGTG ACTCTGACAG TTGGCAGTGT GAACCTAGAG ATCCACAATG GCTTCATCCT 480

CGGGAAGATT CAGCTACCCA GGCCCAAGAT GGCCCCCGCG AATGACACAT ATGAAAGCAT 540

CTTCAGTCAC TTCCGAGAGT ATGAGATTGC CATTCGCAAG GTGCCGGGAA ACTTCACGTT 600

CACACACAAG AAAGTAAAAC ATGAAAACTT CAGCCTCCTA ACCTCTGGAG AAGTGGGAGA 660

GTTCTGTGTC CAGGTGAAAC CATCTGTCGC TTCCCGAAGT AACAAGGGGA TGTGGTCTAA 720

AGAGGAGTGC ATCTCCCTCA CCAGGCAGTA TTTCACCGTG ACCAACGTCA TCATCTTCTT 780

TGCCTTTGTC CTGCTGCTCT CCGGAGCCCT CGCCTACTGC CTGGCCCTCC AGCTGTATGT 840

GCGGCGCCGA AAGAAGCTAC CCAGTGTCCT GCTCTTCAAG AAGCCCAGCC CCTTCATCTT 900

CATCAGCCAG CGTCCCTCCC CAGAGACCCA AGACACCATC CACCCGCTTG ATGAGGAGGC 960

CTTTTTGAAG GTGTCCCCAG AGCTGAAGAA CTTGGACCTG CACGGCAGCA CAGACAGTGG 1020

CTTTGGCAGC ACCAAGCCAT CCCTGCAGAC TGAAGAGCCC CAGTTCCTCC TCCCTGACCC 1080

TCACCCCCAG GCTGACAGAA CGCTGGGAAA CGGGGAGCCC CCTGTGCTGG GGGACAGCTG 1140

CAGTAGTGGC AGCAGCAATA GCACAGACAG CGGGATCTGC CTGCAGGAGC CCAGCCTGAG 1200

CCCCAGCACA GGGCCCACCT GGGAGCAACA GGTGGGGAGC AACAGCAGGG GCCAGGATGA 1260

CAGTGGCATT GACTTAGTTC AAAACTCTGA GGGCCGGGCT GGGGACACAC AGGGTGGCTC 1320

GGCCTTGGGC CACCACAGTC CCCCGGAGCC TGAGGTGCCT GGGGAAGAAG ACCCAGCTGC 1380

TGTGGCATTC CAGGGTTACC TGAGGCAGAC CAGATGTGCT GAAGAGAAGG CAACCAAGAC 1440

AGGCTGCCTG GAGGAAGAAT CGCCCTTGAC AGATGGCCTT GGCCCCAAAT TCGGGAGATG 1500

CCTGGTTGAT GAGGCAGGCT TGCATCCACC AGCCCTGGCC AAGGGCTATT TGAAACAGGA 1560
TCCTCTAGAA ATGACTCTGG CTTCCTCAGG GGCCCCAACG GGACAGTGGA ACCAGCCCAC 1620
TGAGGAATGG TCACTCCTGG CCTTGAGCAG CTGCAGTGAC CTGGGAATAT CTGACTGGAG 1680
CTTTGCCCAT GACCTTGCCC CTCTAGGCTG TGTGGCAGCC CCAGGTGGTC TCCTGGGCAG 1740
CTTTAACTCA GACCTGGTCA CCCTGCCCCT CATCTCTAGC CTGCAGTCAA GTGAGTGACT 1800
CGGGCTGAGA GGCTGCTTTT GATTTTAGCC ATGCCTGCTC CTCTGCCTGG ACCAGGAGGA 1860
GGGCCCTGGG GCAGAAGTTA GGCACGAGGC AGTCTGGGCA CTTTTCTGCA AGTCCACTGG 1920
GGCTGGCCCA GCCAGGCTGC AGGGCTGGTC AGGGTGTCTG GGGCAGGAGG AGGCCAACTC 1980
ACTGAACTAG TGCAGGGTAT GTGGGTGGCA CTGACCTGTT CTGTTGACTG GGGCCCTGCA 2040
GACTCTGGCA GAGCTGAGAA GGGCAGGGAC CTTCTCCCTC CTAGGAACTC TTTCCTGTAT 2100
CATAAAGGAT TATTTGCTCA GGGGAACCAT GGGGCTTTCT GGAGTTGTGG TGAGGCCACC 2160
AGGCTGAAGT CAGCTCAGAC CCAGACCTCC CTGCTTAGGC CACTCGAGCA TCAGAGCTTC 2220
CAGCAGGAGG AAGGGCTGTA GGAATGGAAG CTTCAGGGCC TTGCTGCTGG GGTCATTTTT 2280
AGGGGAAAAA GGAGGATATG ATGGTCACAT GGGGAACCTC CCCTCATCGG GCCTCTGGGG 2340
CAGGAAGCTT GTCACTGGAA GATCTTAAGG TATATATTTT CTGGACACTC AAACACATCA 2400
TAATGGATTC ACTGAGGGGA GACAAAGGGA GCCGAGACCC TGGATGGGGC TTCCAGCTCA 2460
GAACCCATCC CTCTGGTGGG TACCTCTGGC ACCCATCTGC AAATATCTCC CTCTCTCCAA 2520
CAAATGGAGT AGCATCCCCC TGGGGCACTT GCTGAGGCCA AGCCACTCAC ATCCTCACTT 2580
TGCTGCCCCA CCATCTTGCT GACAACTTCC AGAGAAGCCA TGGTTTTTTG TATTGGTCAT 2640
AACTCAGCCC TTTGGGCGGC CTCTGGGCTT GGGCACCAGC TCATGCCAGC CCCAGAGGGT 2700
CAGGGTTGGA GGCCTGTGCT TGTGTTTGCT GCTAATGTCC AGCTACAGAC CCAGAGGATA 2760
AGCCACTGGG CACTGGGCTG GGGTCCCTGC CTTGTTGGTG TTCAGCTGTG TGATTTTGGA 2820
CTAGCCACTT GTCAGAGGGC CTCAATCTCC CATCTGTGAA ATAAGGACTC CACCTTTAGG 2880
GGACCCTCCA TGTTTGCTGG GTATTAGCCA AGCTGGTCCT GGGAGAATGC AGATACTGTC 2940
CGTGGACTAC CAAGCTGGCT TGTTTCTTAT GCCAGAGGCT AACAGATCCA ATGGGAGTCC 3000
ATGGTGTCAT GCCAAGACAG TATCAGACAC AGCCCCAGAA GGGGGCATTA TGGGCCCTGC 3060
CTCCCCATAG GCCATTTGGA CTCTGCCTTC AAACAAAGGC AGTTCAGTCC ACAGGCATGG 3120
AAGCTGTGAG GGGACAGGCC TGTGCGTGCC ATCCAGAGTC ATCTCAGCCC TGCCTTTCTC 3180
TGGAGCATTC TGAAAACAGA TATTCTGGCC CAGGGAATCC AGCCATGACC CCCACCCCTC 3240
TGCCAAAGTA CTCTTAGGTG CCAGTCTGGT AACTGAACTC CCTCTGGAGG CAGGCTTGAG 3300
GGAGGATTCC TCAGGGTTCC CTTGAAAGCT TTATTTATTT ATTTTGTTCA TTTATTTATT 3360
GGAGAGGCAG CATTGCACAG TGAAAGAATT CTGGATATCT CAGGAGCCCC GAAATTCTAG 3420
CTCTGACTTT GCTGTTTCCA GTGGTATGAC CTTGGAGAAG TCACTTATCC TCTTGGAGCC 3480
TCAGTTTCCT CATCTGCAGA ATAATGACTG ACTTGTCTAA TTCATAGGGA TGTGAGGTTC 3540
TGCTGAGGAA ATGGGTATGA ATGTGCCTTG AACACAAAGC TCTGTCAATA AGTGATACAT 3600
GTTTTTTATT CCAATAAATT GTCAAGACCA CA 3632

All references cited herein are incorporated herein by ref-

erence to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

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WHAT IS CLAIMED IS:

1. An isolated or recombinant polypeptide comprising a sequence substitution at position gly351 and/or ser159 or from position leu62 of standard human IL-10 receptor subunit alpha (IL-10RA) and processed forms thereof.

2. The polypeptide of claim 1, wherein:
a) said substitution of gly is with a charged amino acid;
b) said substitution of ser is with an uncharged amino acid; or
c) said polypeptide effects at least a three fold change in signal from IL-10 binding.

3. The polypeptide of claim 2, wherein:
a) said substitution of gly is with arg;
b) said substitution of ser is with gly; or
c) said change in signal is an increase.

4. A method of making an antibody which can distinguish said IL-10RA polypeptide of any one of claims 1 to 3 from the corresponding polypeptide of standard human IL-10RA, comprising immunizing a mammal with said variant, thereby producing an antibody which recognizes said variant but not said standard IL-10RA.

5. A method of making a polypeptide variant of a polypeptide according to any one of claims 1 to 3, comprising expressing an isolated or recombinant nucleic acid encoding said variant polypeptide.

6. The isolated or recombinant nucleic acid of claim 5 encoding said variant polypeptide.

7. A cell transformed with said isolated nucleic acid of claim 6.

8. An isolated or recombinant IL-10RA polypeptide of at least 12 amino acids comprising at least three residues matching each side of and flanking a substitution of standard human IL-10RA at:

a) position gly351 of Seq.ID No.2
b) position ser159 of Seq.ID No.2; or
c) position leu61 of Seq.ID No.5.

9. The isolated polypeptide of claim 8, wherein said:
a) substitution of gly351 of standard human IL-10RA is a super-activating IL-10RA;
b) substitution is G351R;
c) polypeptide matches at least 5 residues on each side flanking gly351
d) substitution is S159G;
e) polypeptide matches at least 5 residues on each side flanking ser159; or
f) substitution of IL-10RA effects at least a three fold difference in signal from IL-10 binding.

10. The polypeptide of claim 8 or 9, comprising a sequence of TLGNREPPV, DTYESIFSH or EVALLSDSD.

11. A method of making a polypeptide of any one of claims 8 to 10, comprising expressing a nucleic acid encoding said polypeptide.

12. An isolated or recombinant nucleic acid encoding a recombinant polypeptide of any one of claims 8 to 10.

13. The nucleic acid of claim 12, further encoding:
a) the extracellular domain of IL-10RA; or
b) C proximal sequence from G351 of IL-10RA.

14. A cell transformed with said nucleic acid of claim 12 or 13.

15. A method of making an antibody which can distinguish said IL-10RA polypeptide of any one of claims 8 to 10 from the corresponding polypeptide of standard human IL-10RA, comprising immunizing a mammal with said variant, thereby producing an antibody which can distinguish.

16. An antibody which can distinguish a polypeptide of

claim 10 from the corresponding polypeptide of standard human IL-10RA.

17. The antibody of claim 15 or 16, which is:
a) a polyclonal antibody preparation;
b) an immunoselected antibody preparation;
c) an immunodepleted antibody preparation; or
d) a monoclonal antibody.

18. A method allowing distinguishing:
a) a nucleic acid encoding said variant of any one of claims 1 to 3 from one encoding standard human IL-10RA, said method comprising comparing said nucleic acid to one encoding said standard human IL-10RA; or
b) a polypeptide variant of any one of claims 1 to 3 from standard human IL-10RA, said method comprising comparing said polypeptides.

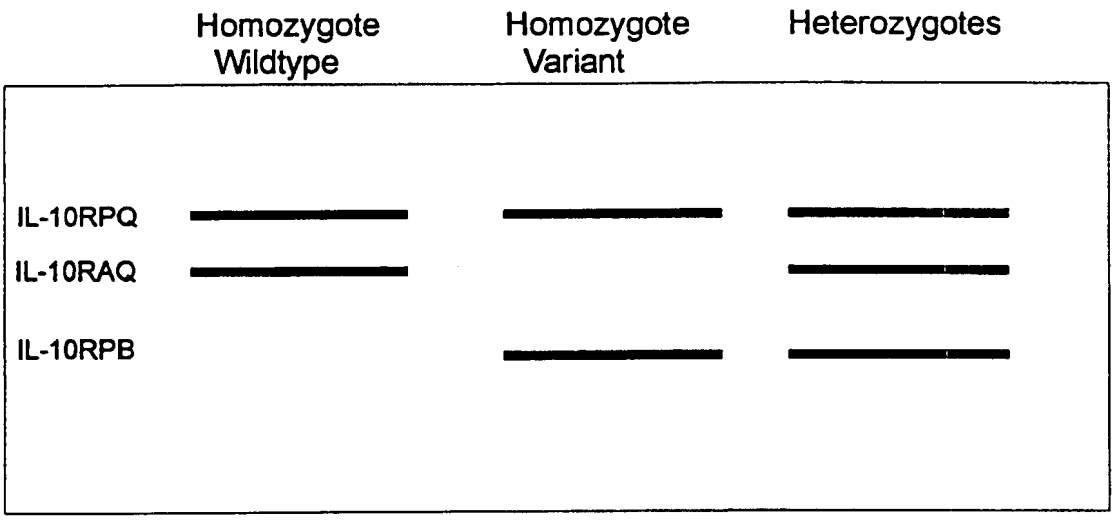
19. The method of claim 18, wherein said comparing is:
a) of nucleic acids, and said comparing is of:
i) PCR products; or
ii) restriction fragments; or
b) of polypeptides, and said comparing is by:
i) immunoassay;
ii) evaluating cell responsiveness to IL-10.

20. The method of claim 18, wherein said distinguishing:
a) allows therapeutic prognosis;
b) provides differential functional information on said respective variants; or
c) determines therapeutic treatment.

21. A kit for performing a method according to any one of claims 18 to 20 comprising
- means for detecting, especially amplifying, nucleic acids encoding standard human IL-10RA,
- means for detecting, especially amplifying, nucleic acids encoding a polypeptide according to any one of claims 1 to 3,
- means for performing detection, especially amplification, of said nucleic acids and,

- optionally, means for detecting the amplified nucleic acids.

Fig.1


Homozygote Wildtype
Homozygote Variant
Heterozygotes
IL-10RPQ
IL-10RAQ
IL-10RPB

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(71) Applicants and
(72) Inventors: **GASCHE, Christoph** [AT/AT]; Babenbergergasse 20, A-3400 Klosterneuburg (AT). **ZAKERI, Schaker, M.** [AT/AT]; Einsiedlerplatz 10/19, A-1050 Vienna (AT).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **REINISCH, Walter** [AT/AT]; Gumpoldskirchnerstrasse 26/3/17, A-2340 Mödling (AT).

(74) Agents: **SONN, Helmut** et al.; Riemergasse 14, A-1010 Vienna (AT).

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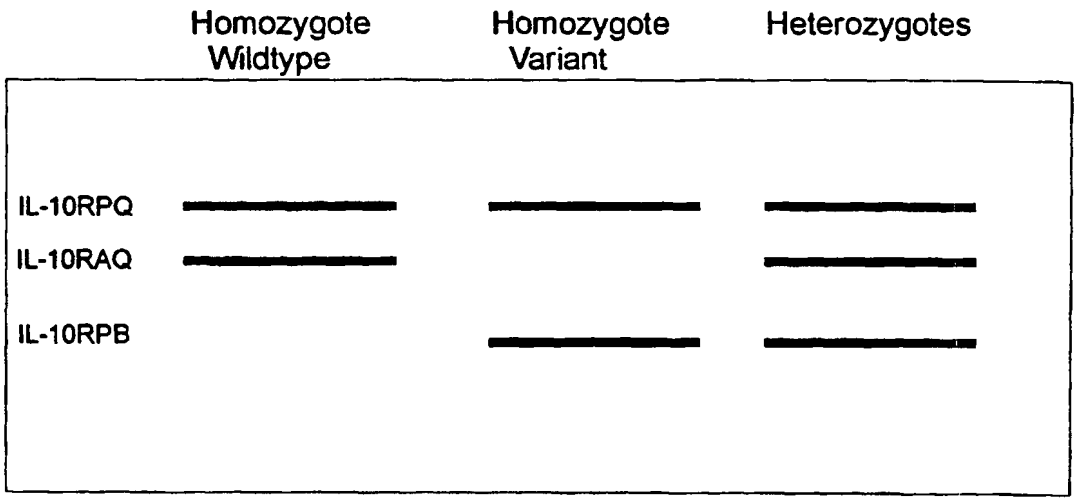
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MAMMALIAN INTERLEUKIN-10 (IL-10) RECEPTOR VARIANTS



(57) Abstract: Amino acid sequence and nucleic acid encoding variants of the IL-10 receptors, including polymorphisms. Uses of the receptor gene and polypeptides are disclosed, including means for screening for agonists and antagonists of the receptor ligands, for producing diagnostic or therapeutic reagents, and for producing antibodies. Therapeutic or diagnostic reagents and kits are also provided.

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INTERNATIONAL SEARCH REPORT

Inte tional Application No
PC /EP 01/02296

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/715 C12N15/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 716 804 A (MOORE KEVIN W ET AL) 10 February 1998 (1998-02-10)	
A	US 5 985 828 A (HSU DI-HWEI ET AL) 16 November 1999 (1999-11-16)	
A	US 5 863 796 A (HSU DI-HWEI ET AL) 26 January 1999 (1999-01-26)	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2002	29/04/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

Form PCT/ISA/210 (second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-21 (in part)

Polypeptide comprising a substitution at position gly351 of human Il-10 receptor subunit alpha. Its uses, antibodies raised against it and nucleic acid encoding therefor,

2. Claims: 1-21 (in part)

Polypeptide comprising a substitution at position ser159 of human Il-10 receptor subunit alpha. Its uses, antibodies raised against it and nucleic acid encoding therefor,

3. Claims: 1-21 (in part)

Polypeptide comprising a sequence substitution from position leu62 of human Il-10 receptor subunit alpha. Its uses, antibodies raised against it and nucleic acid encoding therefor,

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-21 (all partially)

Present claims 1-21 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, a polypeptide of at least 12 amino acids comprising at least three (or at least five) residues flanking a substitution of human IL-10RA or a polypeptide (of any lenght) comprising an (unspecified) sequence substitution starting from position 62 of IL-10RA cannot be considered a permissible generalisation which is fairly based on experimental evidence, that is, it is also not adequately supported by the description. Therefore, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for the general concept underlying the applicaton and for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the IL-10RA specific variants G351R and/or S159G and extended to IL-10RA fragments disclosed in the description, corresponding to seq ID No. 23 and 27 as well as to seq ID No 5.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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